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STUDIES ON EXPERIMENTAL GOITRE. I: THE EFFECT OF BRASSICA SEED DIETS ON RATS.

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THE relationship between iodine deficiency and endemic goitre is now well established, and no factor other than iodine lack has been as yet related to the distribution of endemic goitre in human beings. In experimental animals, however, thyroid hyperplasia has been produced by "goitrogenic" factors, evidently organic substances present in certain vegetable sources. Such positive "goitrogenic" agents have been found in the leaves of the cabbage, and of other Brassicae (Chesney Clawson and Webster, 1928), in Brassica seeds (Hercus and Purves, 1936), and in soy beans (Sharpless, 1938). We consider it of great importance to determine the manner in which such substances produce their effects. This paper reports a further survey of the activity of Brassica seeds, the results of prolonged administration of the active substance, the effect of iodine administration to animals on Brassica diet, and other observations.

MATERIALS AND METHODS.

The seeds of various species of Brassicae were ground and incorporated into diets of the following composition:

Ground seed	45
Skim milk powder	30
Maize meal	20
Marmite	3
Cod-liver oil	1
Sodium chloride	1
	100

The average iodine content of this diet is 300 μg. per kg., which is equivalent to approximately 60μg. of iodine per 1000 calories. Such a diet is relatively rich in iodine. Remington, Remington and Welch (1937) found 60 to 80 μg. per 1000 calories to supply the optimal requirement of the rat.

As test animals we used young albino rats, Wistar strain, 40 to 70 g. in weight, of either sex, the thyroids of both sexes having been found equally susceptible to the action of the diet. At the termination of the experiment

they were killed by coal gas, their thyroids dissected out, weighed, and fixed in formol saline. The specimens taken for histological examination were dehydrated and embedded in the usual manner, sectioned at 4–5μ, and stained with haematoxylin and eosin, or azocarmine and aniline blue.

RESULTS.

Goitrogenic Effects of Diet.

In addition to the seeds previously shown to be active by Hercus and Purves, the following seeds have been shown to contain an active agent: Swede, soft turnip, hard turnip, rape, chou moellier.

Table I shows the result of one experiment made to compare the activity of these seeds.

TABLE I.—*Demonstration of "Goitrogenic" Activity in Brassica Seeds. Three Rats were Fed on Each Species of Seed for 28 Days.*

Diet.	Thyroid weights (mg.).	Histology of thyroid.
Control	10, 11, 13	Colloid storage; normal activity.
Swede seed	31, 41, 43	No colloid; marked hypertrophy and hyperplasia.
Rape seed	35, 37, 45	Ditto.
Soft turnip seed	26, 38, 41	,,
Hard turnip seed	27, 28, 30	,,
Chou moellier seed	43, 49, 55	,,

Chesney Clawson and Webster (1928) found in their experimental rabbits fed on cabbage leaves that there was a progressive enlargement of the thyroid up till 800 days at least, the mean thyroid weight increasing from 0·18 g. to 19·5 g. On analogy with these experiments we expected a progressive enlargement of the thyroids of rats fed on Brassica diets. We therefore put a number of animals on a rape seed diet and sacrificed them at different times.

The results are shown in Table II.

TABLE II.—*Progress of Thyroid Changes on Rape Seed Diet.*

Number of animals.	Time on diet (days).	Average body weights.		Thyroid.		Histology.*
		Initial. (g.).	Final. (g.).	Average weight (mg.).	Range (mg.).	
6	Control	—	116	14·5	12·0–18·0	Normal.
4	7 days	51	68	14·0	12·4–16·3	No colloid; cellular hypertrophy.
4	14 ,,	50	85	33·3	33·3–34·3	No colloid; hypertrophy and hyperplasia.
4	21 ,,	50	90	43·5	36·7–45·5	Ditto.
4	40 ,,	59	109	47·1	42·2–50·0	,,
2	56 ,,	65	136	60·8	57·2–64·4	,,
1	112 ,,	65	137	61·0	—	Some colloid; hypertrophy and hyperplasia.
1	112 ,,	65	127	39·2	—	Ditto.
1	224 ,,	65	204	81·0	—	,,

* Photomicrographs of this series of thyroids are shown in the following paper (Griesbach, 1941).

Effect of simultaneous iodine administration.

In preliminary experiments, rats kept on a goitrogenic diet for 31 days were treated thereafter with amounts of iodine varying from 10 to 1000 μg. daily for 16 days. The iodine was given as potassium iodide, by subcutaneous injection. This treatment was practically without effect on the thyroid. It was decided to determine whether iodine would prevent the thyroid change taking place if this treatment were commenced at the same time as the rats were put on the active diet. Small amounts having proved ineffective, 1000 μg. of iodine were administered daily as potassium iodide.

The results are shown in Table III.

TABLE III.—*The Effect of Iodide Hypodermically on the Thyroid Changes Induced by Rape Seed Diet.*

Number of animals.	Days on rape diet.	Treatment.	Average body weights.		Thyroid.		
			Initial (g.).	Final (g.).	Average weight (mg.).	Range (mg.).	Histology.
2	19	None	70	86	32·9	30·6–35·0	No colloid; marked hypertrophy and hyperplasia.
2	19	1·3 mg. KI daily	65	82	18·4	15·5–21·3	Traces of colloid; marked hypertrophy and hyperplasia.
10	35	None	64	81	30·6	23·4–42·0	No colloid; hypertrophy and hyperplasia.
10	33	1·3 mg. KI daily	65	82	17·2	11·6–21·3	Traces of colloid; hypertrophy and hyperplasia.

Other Effects of Diet.

The diet allows continued growth though at a sub-optimal level. At first it is distasteful to the rats, but once accustomed to it they continue in a healthy condition for as long as the diet has been fed. Post-mortem examinations have been made to detect changes in organs other than the thyroid. Positive results have been observed in three other organs:

(1) There is change in the adrenal cortex, which macroscopically appears pale and opaque. Histologically there is hypertrophy of the cortex with a great increase in the lipoid content of the cortical cells, which accounts for the clay-coloured appearance.

(2) There is a delay in the development of the ovaries of immature female rats. Normal female rats will become pregnant at an age of 2–3 months. Female rats placed on an active diet at the age of six weeks will not become pregnant until an age of 5–7 months.

(3) There are histological changes in the pituitary. These are described in a subsequent paper (Griesbach, 1941).

DISCUSSION.

"Goitrogenic" activity seems to be a general property of the seeds of all Brassica species, as we have so far not tested any which gave negative results.

In general the thyroid hyperplasia induced by so-called goitrogenic agents can be prevented by simultaneous administration of iodine (Webster, 1932; Sharpless, Pearsons and Prato, 1939). The enlargement produced by soy

bean flour could be prevented by a supplementation of 2 μg. of iodine per rat per day. We have found, however, that 1000 μg. of iodine per rat per day failed to prevent entirely the thyroid hyperplasia induced by feeding Brassica seeds, although it markedly diminished the weight response. It is evident that these seeds contain a thyroid activating agent of remarkable potency. The rapid onset (within 7 days) of thyroid changes on our diet is another illustration of the marked potency of these seeds. With soy bean flour, Sharpless (1938) obtained only erratic results at times less than 4 weeks. His results at 7 weeks are comparable with ours at 3–4 weeks.

The progress of the thyroid changes is as follows: In the first 7 days the greater part of the colloid is lost and there is considerable hypertrophy of the epithelium, with large vesicular nuclei, and basophilic changes in the cytoplasm. The gland as a whole is unchanged in size. This is an active secretory phase.

In the second week there is an extremely rapid increase in the weight of the thyroid, from an average of 14·4 mg. to an average of 33·3 mg.—an increase of 138 per cent. Histologically the glands show at fourteen days extreme hypertrophy and hyperplasia, with complete absence of colloid. At the end of the third week the changes are at their maximum. This marks the end of the period of rapid growth, or rapid proliferative phase.

After the third week continued slow thyroid growth accompanies the continued growth of the rat. Colloid, which is absent from the 14th day, reappears again in significant amounts in glands examined subsequent to the 56th day. There is, however, no progressive accumulation of colloid. This would suggest that the thyroid has at this period reached a physiological equilibrium at an increased body-weight ratio.

SUMMARY.

Seeds of Brassicae produce thyroid hyperplasia when fed to rats. This so-called " goitrogenic " activity seems to be a general property of all Brassica species.

The administration of iodine does not prevent the development of thyroid hyperplasia in rats fed on Brassica seeds.

The rapid thyroid proliferation which takes place in the second and third weeks of administration of Brassica seeds is not sustained. After the third week thyroid growth merely parallels the growth of the rat.

Brassica seeds cause hypertrophy of the adrenal cortex, delay in the development of the ovaries of immature females, and histological changes in the pituitary.

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STUDIES ON EXPERIMENTAL GOITRE. II: CHANGES IN THE ANTERIOR PITUITARY OF THE RAT, PRODUCED BY BRASSICA SEED DIET.

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THE relationship between experimental goitre and pituitary cell changes has been known for some time, but there does not seem to be general agreement on the nature of these changes in different animals. Marine, Rosen and Spark (1935), working on rabbits with parenchymatous goitre, considered the acidophil cell type responsible for thyroid stimulation, and they believed that depletion of acidophil granules, coinciding with thyroid activity, suggested identity of these granules with the site of thyrotropic hormone production. Nelson and Warkany (1938, 1939) described marked changes of the basophil cells in pituitaries of rats fed on Steenbock and Black's rachitogenic diet; these changes could be observed after two months' feeding, when the thyroids were hyperplastic, and they proved to be very similar to those following thyroidectomy. Sharpless and Hopson (1940) produced parenchymatous goitre in rats by feeding them on soya bean flour. After 49 days they found the same changes in the pituitary as Nelson and Warkany had found; their evidence was supported by statistically valid cell counts.

It seemed to be of interest to our work on experimental goitre (Hercus and Purves, 1936; Kennedy and Purves, 1941) to search for similar changes in rats fed on the potent goitrogenic Brassica seed diet of Hercus and Purves. For this purpose use has been made of pituitaries from the experimental animals of Purves and Kennedy. The rats had been fed on rape seed diet for varying periods.

TECHNIQUE.

The pituitaries were removed immediately after death and were fixed for four hours in the following solution:

Saturated mercury bichlor. . . .	90 ml.
Formalin	10 ml.

This solution was preferred to Zenker-Formol or Bouin's fluid, since the basophil granules showed a brighter blue and seemed to take the stain more readily. Dehydration was carried through in four hours; the glands were

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then left in cedar-wood oil overnight and embedded in the usual way. Sections were cut at 2·5μ and stained either with Mallory's stain modified by Crossman (1937) or with Heidenhain's "Azan." Unfortunately the Severinghaus (1937, 1938) method could not be used, as "acid-violet" was not available. The Golgi apparatus was stained by Da Fano's technique, counterstaining on the slide with Mallory or Azan. The counterstaining made the determination of the cell types easier. The acidophils took the stain as usual. The "normal" basophils behaved similarly, but the enlarged vacuolated basophils with the giant Golgi body could be stained only occasionally by aniline blue.

RESULTS.

Marked changes were found in the pituitaries as early as seven days after beginning the diet, thus coinciding with the equally rapid changes in the thyroid histology which are described in the previous paper. The nature of the pituitary cell changes may be best presented by considering each cell type separately.

Basophils.

Normally the rat pituitary contains about 4 per cent. of basophils (Sharpless and Hopson, 1940). These "normal" basophils have a relatively homogeneous blue stained cytoplasm, showing very fine granulation. Their nuclei are frequently pyknotic and stain red with Mallory.

7 *days on diet.*—Increased number of "normal" (Fig. 3) basophils; in addition patches of large cells with faint basophil granules, indistinct boundaries and vesicular nuclei, containing 1–2 nucleoli. These cells may, for sake of convenience, be called "goitre cells."

14 *days.*—Changes more marked and uniform (Fig. 5) throughout the gland. Very enlarged basophils, a few of which show patches of deep blue hyalin material in their cytoplasm.

21 *days.*—(Fig. 7) Hyalinization proceeding.

28 *days.*—First appearance of clear vacuoles (Fig. 9) in the blue hyalin, which seems to lie partly in the intercellular spaces. Negative images of enlarged Golgi apparatus in many of the "goitre" cells.

56 *days.*—(Fig. 10) Hyalinization maximal. Many "signet ring" cells and large vacuoles. Basophils now occupy more than half of the section.

112 *days.*—(Fig. 12) Basophil change has now definitely regressed; though many basophils are still present, fewer contain hyalin. Some "signet ring" cells present. It is at this time that colloid is found in the thyroid.

224 *days.*—(Fig. 14) Hyalin has almost disappeared and basophils are mostly of the "negative Golgi" type. There has been some diminution of basophils, but they still cover about one-quarter of the area of the section.

Acidophils.

7–28 days.—There is a considerable loss of acidophils and degranulation in most of them.

56 days.—The hyalinized basophils dominate the picture so largely that only nests of small acidophils can be found.

112–224 days.—(Figs. 12 and 14) Great numbers of acidophils, which are large and contain brilliantly staining granules. The acidophil picture cannot be far from normal.

Chromophobes.

We have found no evidence of changes in this cell type, which sometimes seems to be quite inconspicuous compared with the large basophils. But from the cell counts of Sharpless and Hopson (1940) it may be assumed that the number would be fairly constant.

Golgi apparatus.

We decided to stain the Golgi apparatus, since we were not certain of the classification of the enlarged cells with a rather indistinct granule stain which appear in the early stages. Their Golgi apparatus proved to be of the Severinghaus (1937, 1938) basophil type. After 14 and 21 days it is already larger than the nucleus (Figs. 16 and 17). After 21 and 56 days (Figs. 18 and 19) the Golgi apparatus in the goitre cells is enormous. At the same time the silver impregnation gives a very good impression of the extensive vacuolation.

Mitoses.

Mitotic figures are very rare in normal pituitaries and none have been observed by us in such material. In the pituitaries from Brassica-fed animals three mitoses have so far been noted without any special search being made. These mitoses were all in large basophils of the goitre cell type and show that some at least of these cells arise by proliferation.

DISCUSSION.

There seems to be a strict parallelism between the cellular changes of the pituitaries of rats, as described in this paper and by other authors, and the stimulation of the thyroid. Basophilia is apparent after 7 days with rape seed and at the same date colloid has disappeared from the thyroid. While all the basophil changes increase in degree, hyperplasia of the thyroid goes on to a maximum, reached at about 56 days. From then on the pituitary alteration appears to be more balanced, acidophils increase to nearly normal numbers and size, and the thyroid acini partly fill up with colloid and show flat epithelium. It seemed quite obvious that the basophil changes should correspond to an increased secretion of thyrotropic hormone. On the other hand, reappearance of acidophils, coinciding with colloid storage, seemed to indicate the importance of this cell type for the normal storage function of the thyroid. The latter view would be strongly supported by the work of Marine, Rosen and Spark (1935) on rabbits. But it will be shown in the following paper that the acidophils are at least not indispensable for colloid storage, since this takes place in the hyperplastic thyroid after hypophysectomy. There is no

doubt that acidophils are not responsible for stimulation of the thyroid, since their number is greatly reduced when hyperplasia reaches its maximum. At this stage the enlarged basophils are dominating. Since these cells show a much hypertrophied Golgi apparatus and are seen to undergo mitotic division, they must be in a phase of strong secretion and not, as suggested by some authors for the thyroidectomy cells, in a degenerating condition.

EXPLANATION OF PLATES.

FIG. 1.—Pituitary from control rat showing numerous acidophils, dark in the photograph, occasional granular basophils, and one "signet ring" basophil cell. Mallory. × 450.

FIG. 2.—Thyroid from control rat. H. and E. × 100.

FIG. 3.—Pituitary after 7 days' Brassica diet showing reduction in number and size of acidophils and increase of large basophil cells, pale and granular in the photograph. Mallory. × 450.

FIG. 4.—Thyroid after 7 days' Brassica diet showing cellular hypertrophy and loss of colloid. H. and E. × 100.

FIG. 5.—Pituitary after 14 days' Brassica diet showing large numbers of large pale granular basophil cells and scanty acidophils. (The corresponding thyroid was similar to fig. 8.) Mallory. × 450.

FIG. 6.—Pituitary after 21 days' Brassica diet showing clear hyaline substance accumulating in the large basophil cells. The acidophils are reduced to mere rims of acidophil granules surrounding the nuclei. (The corresponding thyroid was similar to fig. 8.) Mallory. × 450.

FIG. 7.—Pituitary after 28 days' Brassica diet. In addition to the hyaline substance, unstained vacuoles appear in the large basophils. Mallory. × 450.

FIG. 8.—Thyroid after 28 days' Brassica diet, showing extreme degree of cellular hypertrophy. H. and E. × 450.

FIG. 9.—Pituitary after 56 days' Brassica diet. Extreme accumulation of hyaline substance in the large basophils. Some of the hyaline substance appears to be intercellular. Many unstained vacuoles present in both hyaline substance and basophil cells. Mallory. × 450.

FIG. 10.—Pituitary after 112 days' Brassica diet. Considerable regression of the large basophils has taken place. Many of the remaining basophils have taken the form of "signet ring" cells, a thin rim of cytoplasm enclosing a mass of vacuolated hyaline substance. There is an increase in the number of acidophils. Mallory. × 450.

FIG. 11.—Thyroid after 112 days' Brassica diet. Colloid has reappeared in the acini. H. and E. × 100.

FIG. 12.—Pituitary after 224 days' Brassica diet. Acidophils have returned almost to normal numbers. The basophils show well-marked negative images of the Golgi apparatus. Mallory. × 450.

FIG. 13.—Thyroid after 224 days' Brassica diet. The colloid has not increased since 112 days. H. and E. × 100.

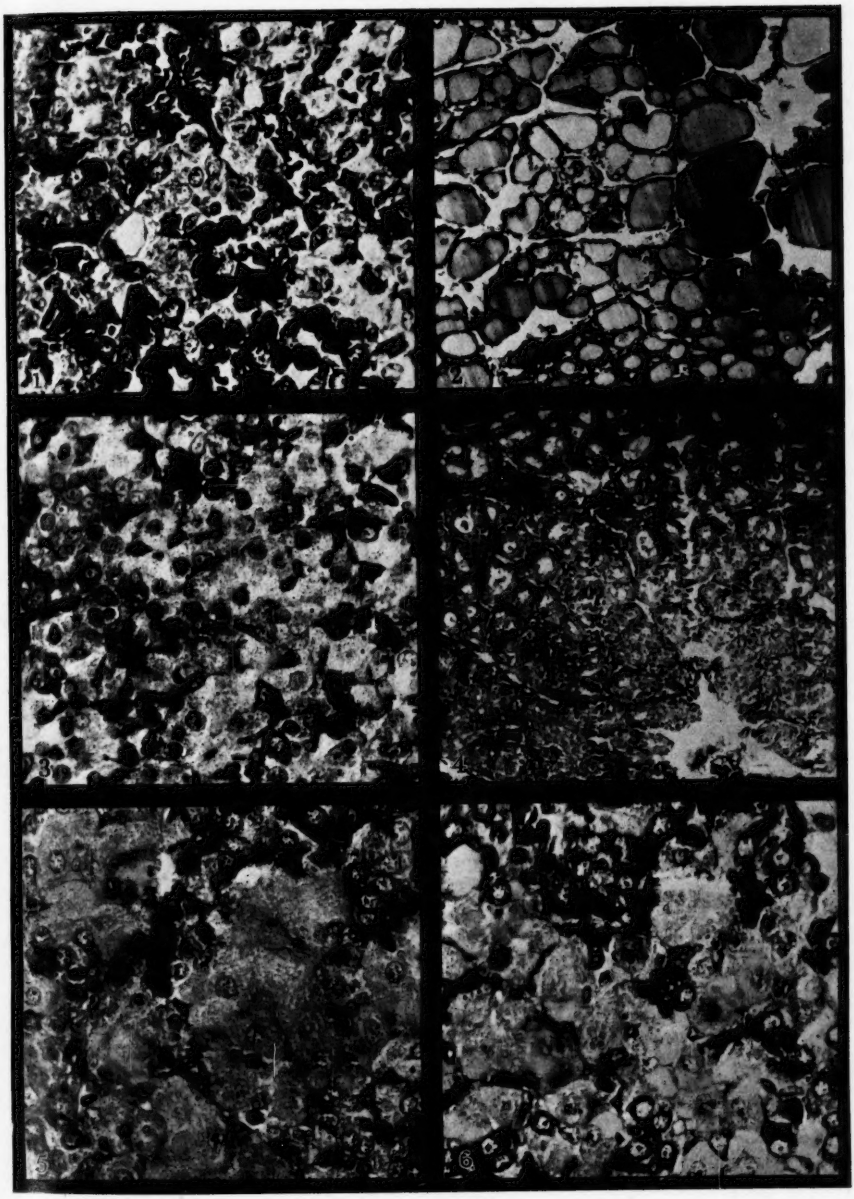
FIG. 14.—Pituitary after 14 days' Brassica diet. Da Fano preparation showing Golgi bodies delicately impregnated with silver. The Golgi bodies of the large basophils ("goitre cells") are larger than the nuclei which they partly embrace. Da Fano + Mallory. × 450.

FIG. 15.—Pituitary after 21 days'. Brassica diet. Similar to fig. 15, but the silver impregnation has been denser and the Golgi bodies appear black. Da Fano + Mallory. × 450.

FIG. 16.—Pituitary after 56 days' Brassica diet. Da Fano preparation showing the hypertrophic Golgi body of a large basophil ("goitre cell"), the cytoplasm of which is extensively vacuolated. Da Fano + Mallory. × 1000.

FIG. 17.—The same as fig. 17. Adjacent to the large "goitre" cell are two "normal" basophils with smaller Golgi bodies. Da Fano + Mallory. × 1000.

FIG. 18.—Pituitary after 112 days' Brassica diet. A typical "signet ring" cell and several acidophils. Azan. × 1000.



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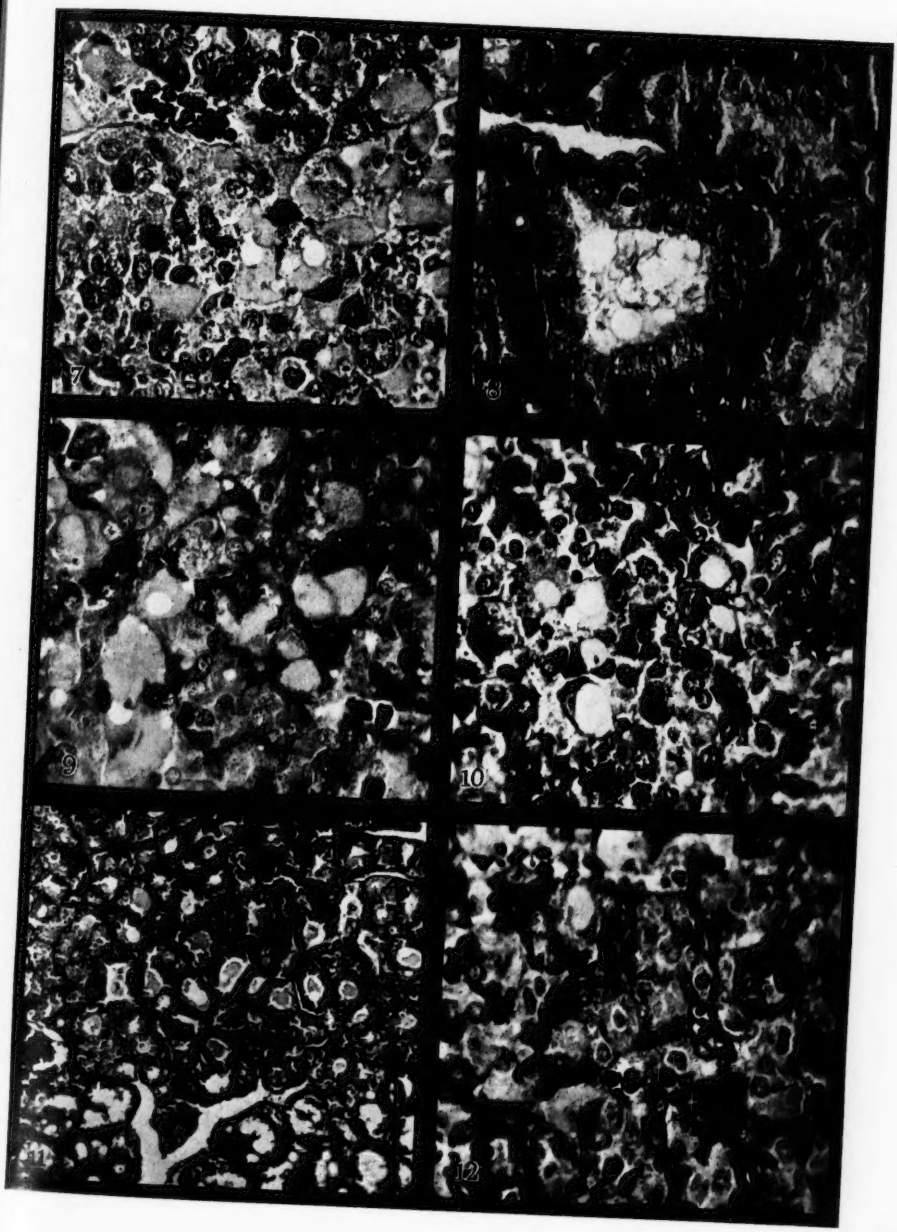

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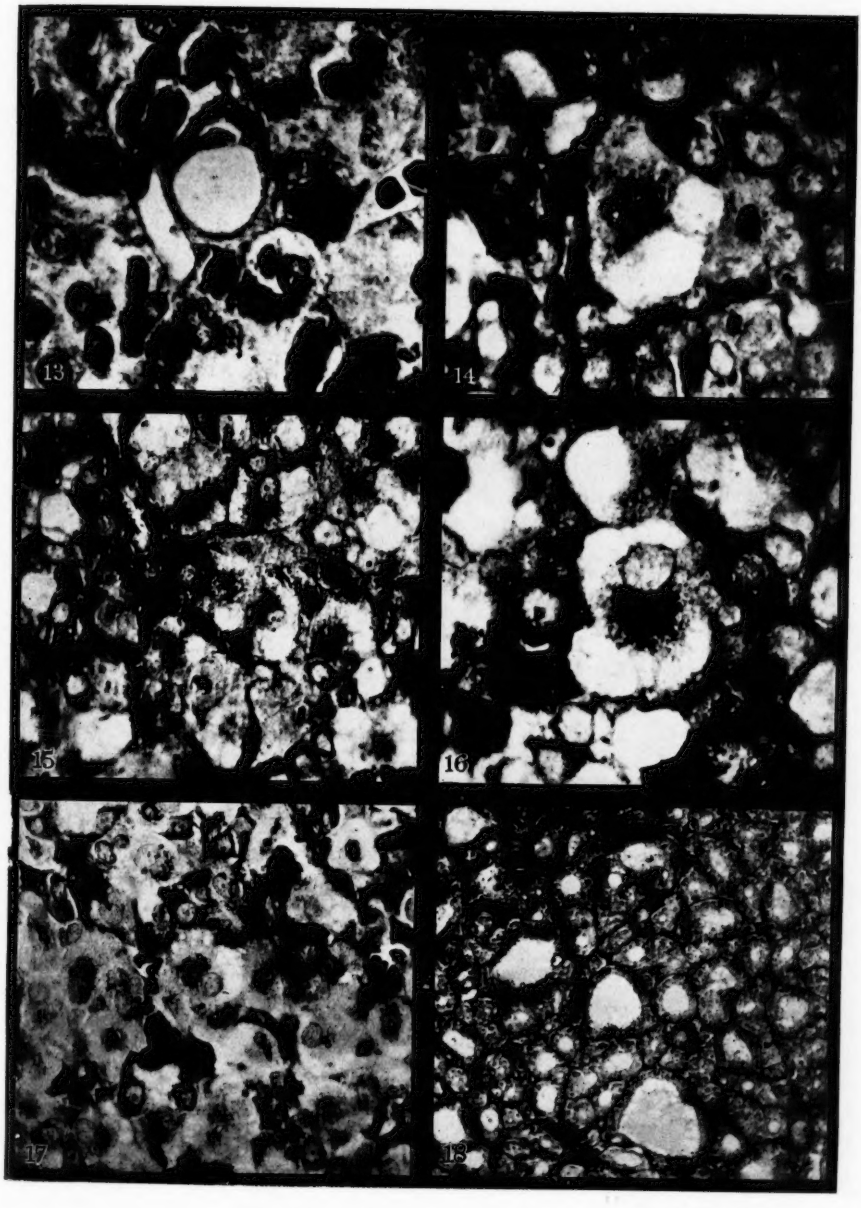

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SUMMARY.

Cellular changes are described in the pituitaries of rats fed on rape seed "goitrogenic" diet. The changes consist of a remarkably rapid increase in basophil cells with hyalinization, vacuolation, formation of "signet ring" cells and a simultaneous loss of acidophils. After reaching a maximum at 56 days a more normal picture appears, coinciding in time with reappearance of colloid in the hyperplastic thyroids. The results are consistent with the hypothesis that the basophils are the source of thyrotropic hormone.

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STUDIES ON EXPERIMENTAL GOITRE. III: THE EFFECT OF GOITROGENIC DIET ON HYPOPHYSECTOMIZED RATS.

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IN previous papers (Kennedy and Purves, 1941; Griesbach, 1941) experiments were described showing the effect of rape and other Brassica seeds on the thyroids and pituitaries of rats. The close relationship between thyroid hyperplasia produced by feeding these seeds and cytological changes in the anterior lobe of the pituitary has been demonstrated, thus confirming and amplifying the work of Nelson and Warkany (1938) and Sharpless and Hopson (1940) published while our experiments were in progress. With the data

available it is not possible to decide on the point of action of the active principle, because both thyroid and pituitary are affected simultaneously as early as seven days after starting the rape seed diet. Since thyroidectomy alone produces pituitary changes so similar to those of goitrogenic diet, the removal of the thyroid does not appear likely to settle the question. Hypophysectomy, however, by excluding the mediation of the thyrotropic hormone, would make it possible to detect any direct stimulating action of the active "goitrogenic" principle on the thyroid gland. Therefore we decided to examine the influence of goitrogenic diet upon the thyroids of hypophysectomized rats. The Brassica seeds appeared to be particularly suitable for such experiments, since the time required is much shorter than with any other known agent—an important consideration in work on hypophysectomized animals.

MATERIALS AND METHODS.

Young rats of the Wistar strain weighing 80–120 g. were used. Males were preferred latterly, as the atrophy of the testicles is a convenient sign of complete hypophysectomy.

Operation.

The operation was at first performed by the parapharyngeal method as described by Thompson (1932). After some experience had been obtained modifications of the procedure were tested out, leading to a modified technique which we found to be easier of performance and to give better survival rates. The deviations from Thompson's technique are as follows: The digastric muscle was left intact, and the base of the skull was approached by separating the omohyoid muscle from the sternothyroid, etc., by blunt dissection with fine forceps. The larger vessels were thus avoided, and there was only occasional bleeding from small muscle arteries. This approach is much nearer to the midline and allows the use of a new retractor, constructed by one of us (H. D. P.) and shown in Fig. 1. The retractor, which is introduced after mobilization of the pharynx from its attachment to the basi-occiput, is used to retract the hyoid bone anteriorly and the pharynx to the operator's left across the mid-line, thus exposing the skull base. This instrument is very useful, since it protects the pharynx from perforation, which is invariably fatal, and at the same time reflects so much light that no special illumination apart from an ordinary desk lamp, is necessary. It is not possible, however, to leave the retractor in position for more than a short time, as it obstructs respiration. Cessation of respiration was treated by giving the rat forced inhalation through the nose with a Higginson's syringe connected with a short tube. By this means deaths due to asphyxia were entirely prevented, and the preliminary tracheotomy which is advised by some writers rendered unnecessary. After using the dental drill, as described by Thompson, in a large number of operations, we found it more satisfactory to use two sizes of hand-drills, shown in Fig. 1. The smaller drill with the spear point was employed for accurately marking and starting the hole about 1 mm. in front

of the synchondrosis. The second, blunter instrument was used for continuing the hole through the bone. If necessary, an enlargement was made with a 9–10 dental burr, mounted on a handle and fitted with a small metal collar, to regulate the depth of penetration. By these means the more delicate work was under finer control, and no haemorrhage occurred in most cases. Pieces of bone from the inner layer of the skull should be carefully picked, as described by Thompson, but we did not always succeed in doing this completely. Our retractor greatly facilitated the visual control of the removal of the pituitary by suction. The time required to operate in this way did not exceed 20 minutes.

Anaesthesia.

Nembutal (35 mg. per 100 g. rat intraperitoneally) was used as anaesthetic and the anaesthesia was deepened when necessary by the inhalation of a few drops of ether. We found it very useful to give atropine sulphate to diminish mucus production in the trachea. For this purpose we combined the atropine and nembutal in the following solution :

Nembutal	0·10 g.
Atropine sulph.	0·04 g.
Saline to	10 ml.

Diet.

The goitrogenic diet used in these experiments had the following composition :

	Per cent.
Rape seed, crushed	45
Skim milk powder	30
Maize meal	20
Marmite	3
Cod-liver oil	1
Sod. chlor.	1

Great difficulty was experienced in persuading the hypophysectomized rats to take the goitrogenic diet. Moreover, rats which had remained in good condition after hypophysectomy for 2 to 3 weeks on stock diet became seriously ill after changing to the active diet. They refused food and water and developed spasticity, bradycardia and subnormal temperature, and eventually died in convulsions. Since these symptoms resembled the B_1 deficiency syndrome, we gave massive doses of B_1 in the form of marmite and B_1 adsorbate (old international standard) added to the diet and the drinking water. This was followed by a striking improvement in the condition of the animals. They lost their spasticity and took their food as well as the healthy ones did. Under this régime the survival rate was satisfactory, though a certain mortality is always to be expected. Incidentally, the above mentioned symptoms never developed in incompletely hypophysectomized animals.

RESULTS.

Two series of experiments were made. In the first, rats were hypophysectomized, kept for 2 to 3 weeks on stock diet, and were then transferred to active diet. They were killed after varying periods up to 91 days, the thyroids were dissected and weighed and fixed for histological examination. The skull was opened and the base examined carefully for pituitary remnants. Since in those animals judged to be completely hypophysectomised the thyroids were atrophic, it was unnecessary for us to make serial sections of the base of the skull to detect possible pituitary remnants. The results of this series are shown in Table I, A and B.

TABLE I.

	No.	Days on diet.	Weight change after operation (g.).	Thyroid. Weight in mg.	Thyroid. Histology.
A. Completely hypophysectomized	1	14	−12	6·0	Well stained colloid—flat epithelium.
	2	54	−17	6·0	Ditto.
	3	54	−34	5·4	,,
	4	56	−24	5·2	,,
	5	56	−15	4·9	,,
	6	63	−26	6·4	,,
	7	91	−48	5·0	,,
B. Incompletely hypophysectomized	8	33	+20	19·7	Some poorly stained colloid—high epithelium.
	9	42	+ 6	18·0	Ditto.
	10	42	+11	22·0	,,
	11	51	+12	33·0	No colloid—high epithelium.
	12	60	+33	29·7	Ditto.
	13	63	0	28·8	,,
	14	91	+52	29·0	,,
C. Unoperated controls	15	35	+40	44·0	,,
	16	38	+32	34·2	,,

EXPLANATION OF PLATE.

FIG. 1.—Instruments used for hypophysectomy. The handles were from old dental instruments. *a*. View of retractor from below, showing J-shaped lower end which contacts the base of the skull, the broad left surface protecting the pharynx. *b*. View of retractor from behind. *c*. Spear-pointed drill. *d*. Blunt drill. *e*. Dental burr mounted on handle with collar to limit penetration.

FIG. 2.—Thyroid from completely hypophysectomized rat given Brassica diet, Experiment I. H. and E. × 100.

FIG. 3.—Thyroid from incompletely hypophysectomized rat given Brassica diet, Experiment I. H. and E. × 100.

FIG. 4.—Thyroid from completely hypophysectomized rat given Brassica diet before and after operation. H. and E. × 100.

FIG. 5.—Thyroid from incompletely hypophysectomized rat given Brassica diet before and after operation. H. and E. × 100.

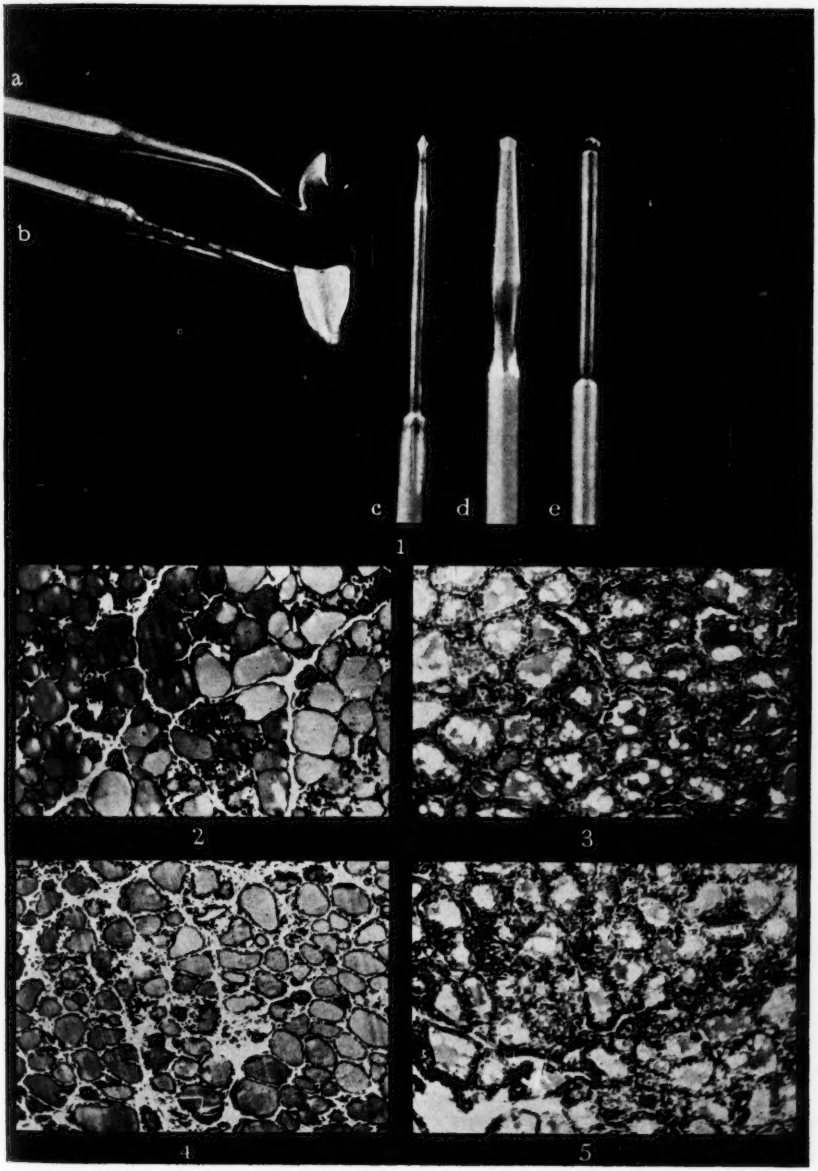

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TABLE II.

	No.	Days on diet after operation.	Weight change after operation (g.).	Thyroid. Weight in mg.	Thyroid. Histology.
A. Completely hypophysectomized	1	20	−36	9·4	Well-stained colloid—flat epithelium.
	2	21	−34	11·9	Ditto.
	3	21	−29	13·6	,,
	4	22	−23	10·4	,,
	5	22	−38	15·3	,,
	6	23	−26	13·7	,,
	7	23	−28	12·8	,,
B. Incompletely hypophysectomized	8	21	− 3	26·6	No colloid—high epithelium.
	9	23	+17	40·0	Ditto.

In the second series, rats aged 6 weeks were placed on active diet for 51 to 53 days. They were then hypophysectomized, the presence of an enlarged hyperplastic thyroid being verified during the initial stages of the operation. In this series the animals came out of the anaesthetic (nembutal) much more rapidly than those of the previous series. These animals were killed 20 to 23 days after hypophysectomy and examined as for the first group. The results are shown in Table II.

It will be seen in Table I that the completely hypophysectomized animals had small thyroids weighing from 4·9 to 6·4 mg. These glands were pale and translucent, in marked contrast with the enlarged dark red thyroids found in the incompletely hypophysectomized animals. The thyroid glands of the latter, weighing from 18 to 33 mg., were considerably greater than the normal thyroid weight of rats of this size (11·3 mg.), but were less than those of unoperated rats on this diet.

The histology of the thyroids in Table I, A, always showed the picture of the resting gland with very flat epithelium, oval nuclei, and acini of varying size filled with well staining colloid (Fig. 2). The histological structure of the thyroids in incompletely operated rats showed different grades of activity, but this was not as high as in the unoperated controls. Although the epithelium was always columnar, numerous acini contained colloid at a time when depletion would be expected (Fig. 3). The activity of the thyroids probably corresponds to the amount of pituitary left behind. This varied from one anterior lobe to a mere crescent estimated at one sixth of one anterior lobe. Even this small remnant was able to effect a marked stimulation of the thyroid.

Table II gives evidence of the effect of hypophysectomy on the thyroids of rats previously fed on active diet, and being kept on that diet after operation. The thyroids of rats 1 to 7 were pale and translucent, their weights 9·4 to 15·3 mg. The change in appearance and sizes, checked during operation, was striking, considering that the estimated weight was 25 to 50 mg. and the colour had been the typical dark red of the active gland. The histology of all these thyroids was the same, proving inactivity (Fig. 4). In rats 8 and 9 we found

remnants of the pituitary. The thyroid weight was 26·6 and 40·0 mg. respectively and high activity was found (Fig. 5).

DISCUSSION.

It is clear that the rape seed diet produces no activation of the thyroid in the completely hypophysectomized rat. The thyroid hyperplasia, previously produced, rapidly regresses after hypophysectomy, despite the continuous administration of the active principle.

From these results it is clear that the active principle of Brassica seeds cannot stimulate the thyroid directly, but requires the mediation of the thyrotropic hormone. These experiments, however, do not determine whether the sensitivity of the thyroid to physiological amounts of thyrotropic hormone is increased by the goitrogenic factor, or the pituitary stimulated to secrete abnormal amounts of this hormone. There is indirect evidence, however, notably in the contemporaneous histological changes in the pituitary, which suggests that the latter view is the correct one.

A point of interest is the rapid reappearance of colloid in the thyroids of the animals of experiment II (Table II, A). At the time of hypophysectomy these thyroids would be free from all but traces of colloid. Three weeks later they were full of colloid. This shows that the thyroid, even while under the influence of the "goitrogenic" diet, is capable of forming and storing well staining colloid when the pituitary is absent and the stimulating effect of the thyrotropic hormone removed. We have confirmed this formation of colloid after hypophysectomy in five animals in which the absence of colloid from the thyroid was verified by the removal of one lobe for histological examination previous to the removal of the pituitary.

SUMMARY.

The "goitrogenic" principle of Brassica seeds does not directly stimulate the thyroid. To produce thyroid hyperplasia it requires the mediation of the thyrotropic hormone of the pituitary.

Hyperplasia previously induced by Brassica feeding rapidly regresses after hypophysectomy despite continued feeding of the Brassica seeds.

Colloid formation and storage take place during involution of the thyroid after hypophysectomy, and this colloid formation is not prevented by the "goitrogenic" principle of Brassica seeds.

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PURIFICATION OF DIPHTHERIA TOXIN AND TOXOID MADE FROM TRYPTIC DIGEST BROTHS.

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In 1936 Eaton was able to isolate, from culture filtrates of *C. diphtheriae* grown on a proteose peptone medium, a protein which appeared to be pure, or almost pure, diphtheria toxin. In his paper Eaton (1936) gives an extensive and critical review of the earlier literature on this subject, and therefore such a review will not be attempted here. Eaton's findings were confirmed by Pappenheimer (1937) using a gelatin hydrolysate medium, and these workers agree that pure diphtheria toxin is a heat coagulable protein containing 0·00046 mg. nitrogen per Lf unit, or 2146 Lf units per mg. nitrogen. Both their procedures are somewhat lengthy and laborious, but Eaton and Gronau (1938) have recently described a much simpler method by which the toxin can be obtained reasonably pure. Although still containing some nitrogenous impurities, such preparations appear to be sufficiently pure for many biological purposes, and, what is more, the toxin suffers little or no damage during this purification. As Eaton (1936) and Pappenheimer (1937) have stated, if undamaged toxins are required the toxin solutions must be kept between pH 6·0 and 9·0, otherwise it will be found that the flocculating ability of the toxin is impaired and loss of toxicity will occur. These conditions should be borne in mind for the purification of toxoid also, and the writer can confirm the work of Schmidt (1930) and Leonard and Holm (1933), who have found that any method of purification involving acid precipitation at a pH below 6·0 has invariably resulted in an increase in the time of flocculation of the toxoid. As is the case for toxin, an increase in the time of flocculation is a sure sign of damage to the toxoid protein.

Most of the toxins and toxoids in use at these laboratories are prepared from the weak tryptic digest broth described by Pope and Linggood (1939). This broth has a nitrogen content of 2·0 to 2·4 mg. per c.c. and, with the strain of Park Williams 8 in use, growth occurs principally on the surface of the medium, the formation of " curtains " and heavy deposits being reduced to a minimum. The Lf value of toxins from such broths is 35 to 40 units per c.c.

Eaton used a Difco proteose medium and it has been found necessary to modify his method somewhat for the purification of our toxins. On the addition of one-fifth volume of 5 per cent. cadmium chloride solution to the toxin (to which 2 per cent. of solid sodium citrate has been added) no immediate precipitate of insoluble phosphate results. A faint opalescence may occur,

and the mixture has to be left for 24 hours, at least, in the cold room (4° C.) before the reaction appears to be complete. After this period a heavy precipitate has formed, but it was found that considerably more 5 per cent. cadmium chloride solution has to be added before the phosphates present in the toxin are completely eliminated. With most of our toxins (not toxoids, however) up to 30 c.c. of 5 per cent. cadmium chloride solution per 100 c.c. of citrated toxin can be added without an appreciable loss of toxin and before an excess of the reagent appears in the filtrate. The position of the end-point can be readily found by the determination of the Lf value of the filtrates. Until excess cadmium chloride appears in the filtrates, the Lf values of the latter are equal to, or only slightly less than, the value which would be expected as the result of the dilution of the toxin with the cadmium chloride solution. As soon as an excess of the reagent occurs in the filtrate, however, the Lf value begins to fall rapidly away from the expected value. It has been found advisable to determine this optimal amount of cadmium chloride, for if too little is added, the bulk of the precipitate in the second precipitation, i.e. that of the toxin itself, may become unnecessarily large, with the result that the purity of the product is diminished.

Again, with our toxins after the removal of the phosphate precipitate, the addition of one-third volume of 5 per cent. cadmium chloride solution results in the formation of a heavy flocculent precipitate, which goes partially into solution at pH 6·0, but remains flocculent ; the slow formation of a granular precipitate, as described by Eaton, has not been encountered. It was not found necessary to add as much as one-third volume of cadmium chloride solution to precipitate all the toxin ; smaller quantities would suffice, and as this resulted in the precipitation of the toxin in a smaller bulk of precipitate, it was considered advisable to use the minimal quantity of 5 per cent. cadmium chloride solution that would just precipitate all, or almost all, the toxin in question.

Having secured satisfactory results with our toxins it was thought to be of interest to see if the method could be applied to toxoids. With toxoid the precipitation of the phosphates does not proceed quite so smoothly, and more toxoid is lost in this initial stage than is the case with toxin, but by leaving the mixtures for 48 hours in the cold room, it is possible to determine the maximal quantity of cadmium chloride that can safely be added without undue loss of Lf units. This end-point is more definite after 48 hours in the cold room than after 24 hours. Apart from this slight difficulty, the method was found to work quite well for toxoids.

Pope and Linggood (1939) and Linggood (1939) have shown how the purity of toxoids from their weak tryptic digest broths may be improved by treatment with a brand of charcoal known as " Eponit." Not only does the purity, as expressed in terms of Lf units per mg. nitrogen, increase after such treatment, but also the heat coagulable protein nitrogen can be considerably reduced. These workers have expressed the purity of their preparations as a percentage ratio, i.e. the ratio in per cent. of the theoretical heat coagulable nitrogen of the toxin or toxoid as calculated from Pappenheimer's figure (2146 Lf units per mg. nitrogen) to the heat coagulable nitrogen actually found by experiment ; by such means an idea of the extent of the protein impurities present

in a toxin or toxoid may be obtained. Thus it was thought to be of interest to determine the purities that could be produced by the cadmium chloride method of purification from charcoal treated toxins and toxoids, since charcoal treatment is rapidly and easily carried out and is unlikely to damage the toxin or toxoid protein.

EXPERIMENTAL.

The following technique has been adopted for the purification of toxins and toxoids from our weak tryptic digest broths. Two per cent. of solid sodium citrate is added to the crude or charcoal treated toxin or toxoid, and to 50 c.c. samples varying amounts of 5 per cent. cadmium chloride solution, from 10 c.c. to 17·5 c.c., are added. The mixtures are then left in the cold room (4° C.) for 24 hours in the case of toxin and 48 hours when toxoid is being used. Each mixture is then filtered and the Lf value of the filtrate determined. Typical results are shown in Table I. From this table it will

TABLE I.—*Removal of Phosphate with Cadmium Chloride from Citrated Toxins and Toxoids.*

Nature.	Lf.	Volume $CdCl_2$ per 50 c.c. (c.c.).	Lf possible in filtrate units/c.c.	Lf. found in filtrate units/c.c.
Crude toxin	38·5	10·0	32	31·5
		12·5	31	29·5
		15·0	29·5	26·5*
		17·5	28·5	21·5
Crude toxoid	37·5	10·0	31	27·5
		12·5	30	25·5*
		15·0	29	15·5
		17·5	28	8

* Amount chosen as most suitable.

be seen that it is easy to decide on the maximal amount of 5 per cent. cadmium chloride which can be safely added to the bulk of the citrated toxin or toxoid.

After the latter mixture has stood for the appropriate time in the cold room, the precipitate is removed by filtration and the amount of 5 per cent. cadmium chloride solution required to precipitate all, or almost all the toxin in the filtrate is determined. 50 c.c. samples are taken and amounts of 5 per cent. cadmium chloride, varying from 5 c.c. to 17·5 c.c., are added.

A flocculent precipitate forms in all mixtures, which are then adjusted to pH 6·0 (glass electrode) with 25 per cent. hydrochloric acid. A visible diminution in the size of the precipitate should occur, and with the smaller amounts of cadmium chloride the precipitate may go into solution completely. The mixtures are left in the cold room overnight. Next day they are filtered and the Lf value of the filtrate determined. This is done by blending the filtrate with a quick flocculating toxin of known titre according to the method of Glenny, Pope, Waddington and Wallace (1926). To prevent precipitation

of the blend toxin with the excess cadmium chloride in the filtrates it is necessary to add approximately 6 per cent. of solid sodium citrate followed by a few drops of strong caustic soda to bring the pH of the filtrates up to pH 8·0 approx. By this means it is possible to determine quite readily whether the Lf value of the filtrate is zero, two, four or more units.

After the amount of 5 per cent. cadmium chloride solution which will just precipitate the toxin or toxoid at pH 6·0 has been determined, the bulk of the filtrate is treated with the calculated amount of the reagent and the mixture adjusted to pH 6·0 with 50 per cent. hydrochloric acid. A small flocculent precipitate is left. This is allowed to settle in the cold room overnight and the next morning the clear supernatant is decanted. The precipitate is collected and washed four times with saline by centrifugalization, or, if preferred, it may be filtered and washed on a Büchner funnel. The latter method is occasionally better when working with the precipitate from crude toxoids, as these precipitates sometimes fail to give clear supernatants when centrifuged.

The purified toxin or toxoid can then be eluted from the precipitate by leaving overnight in the cold room with 2 per cent. disodium hydrogen phosphate ($Na_2HPO_4.12\ H_2O$), the mixture being adjusted, if necessary, to pH 7·8–8·0. One-twentieth of the original volume of the toxin or toxoid is the volume of phosphate usually taken for the elution. In this manner concentrated toxins and toxoids of high purity can be obtained. Those from crude toxins and toxoids are reddish brown in colour, while those from charcoal-treated preparations are almost colourless, at the most a pale straw colour. The results obtained by this method are shown in Table II.

DISCUSSION OF RESULTS.

An examination of Table II reveals the fact that, in spite of the variability in the purities obtained, the latter are quite good when it is remembered that the toxin or toxoid has been precipitated once only, at a pH not below 6·0. The beneficial effect of charcoal treatment, both with toxin and toxoid, is clearly demonstrated, and the greatly increased purity of the products is well worth the loss of Lf units which such a procedure involves. Another great advantage of the method of cadmium chloride purification over many others is clearly shown in Table II, where it will be seen that the purified products flocculate as quickly, if not more quickly, than the corresponding unpurified toxin or toxoid—a sign that little or no damage has been done to the specific proteins.

It is difficult to compare these results with the purities obtained by Eaton and Gronau (1938), who used a proteose peptone broth for the production of their toxin, for they quote the purities obtained by a single precipitation with cadmium chloride for two toxins only and express their results as the nitrogen contained in a m.l.d. of toxin. Moreover the relationship between the m.l.d. and Lf may vary from toxin to toxin. Eaton in an earlier paper (1936) states that in his most pure preparations the ratio m.l.d. per c.c. to Lf units per c.c. is between 20 and 35, while Pappenheimer (1937) gives the figure of

TABLE II.—*Showing the Purities Obtai*

TABLE II.—*Showing the Purities Obtained with Toxins and Toxoids by a Single Precipitation with Cadmium Chloride.*

	Original motarial.				Purified product.				
					Dilution tested.				
Nature.	No.	Charcoal per cent.	Lf units/c.c.	Kf 50° C. (mins.).	Nitrogen mg./c.c.	Lf units/c.c.	Kf 50° C. (mins.).	Lf units/c.c.	Nitrogen mg./c.c.	Purity Lf/mg. N.	Yield per cent.*
Toxin	512	None	35·5	11	2·32	41	7	82	0·127	646	69
,,	514	,,	29·5	12	..	30·5	13	152·5	0·193	790	54
,,	519	4	29	10	..	31	..	222	0·145	1530	50
,,	526	4·5	33	12	..	39	7	390	0·314	1240	52
,,	532	2·5	31	11	..	29	10	290	0·202	1430	65
,,	540A	None	45	10	2·27	43	8	430	0·762	565	59
,,	540B	2	37·5	10	1·53	28·5	10	285	0·373	764	50
,,	540C	4	26·5	16	1·12	45	8	225	0·240	940	54
,,	550	4	25·5	15	..	33·5	11	268	0·234	1150	56
,,	566	2	26·5	16	1·34	30·5	10	305	0·238	1280	65
,,	567A	None	38·5	14	1·93	37	11	296	0·295	1002	62·5
,,	567B	2	32·5	12	1·34	33	10	330	0·263	1250	61
,,	567C	3	28·5	15	1·09	35·5	7	355	0·237	1500	64
,,	573A	None	38·5	14	2·14	26·5	18	265	0·274	966	64
,,	573B	3	26·5	12	1·02	42	8	210	0·138	1520	62
Toxoid	1612A	None	38·5	24	2·19	41	18	410	0·713	575	64·5
,,	1612B	3	32·5	19	1·15	47	14	470	0·437	1070	66
,,	1612C	5	27·5	24	0·733	32	19	320	0·222	1440	55
,,	1737	5	21·5	29	..	53	12	530	0·447	1180	39†
,,	1739	4	27	22	..	43	15	430	0·466	923	60
,,	1768A	None	37·5	29	2·00	34·5	30	345	0·613	565	67·5
,,	1768B	5	23·5	20	0·632	51	10	255	0·168	1520	50‡

* Excluding charcoal treatment.
† Toxoid not completely precipitated. Lf value of supernatant = 5·5 units per c.c.
‡ ,, ,, ,, ,, ,, ,, ,, = 3·5 ,, ,,
N.B.—All dilutions of purified products made in 0·9 per cent. saline."

TABLE III.—*Showing the Superiority of the Cadmium Chloride Method of Purification over that Using Willstätter Cream.*

Toxin PT 567.

				Cadmium chloride method.			Willstätter cream method.		
Charcoal per cent.	Lf units per c.c.	Nitrogen mg. per c.c.	Ratio.*	Purity. Lf units per mg. N.	Ratio per cent.*	Yield per cent.	Purity. Lf. units per mg. N.	Ratio per cent.*	Yield per cent.
None	38·5	1·93	43	1002	..	62·5	394	..	51
2	32·5	1·34	60	1250	85	61	555	85	52·5
3	28·5	1·09	65	1500	99	64	708	91	53·5

* See Pope and Linggood (1939).

30 for this relationship. If, for the purposes of calculation, the figure of 30 is taken, then the purities of the two products quoted by Eaton and Gronau work out to be 330 Lf units per mg. nitrogen and 760 Lf units per mg. nitrogen—figures comparable with those obtained with our toxins.

It was thought to be of interest to compare these results with those obtained by the Willstätter cream method of purification. The results are given in Table III, and show clearly the undoubted superiority of the cadmium chloride method. This superiority is due, it is thought, firstly to the comparatively large amounts of Willstätter cream that have to be used in order to adsorb completely, or almost completely, the specific toxin, and secondly to the fact, as Eaton has postulated, that the cadmium proteose complexes are soluble in citrate at pH 6·0.

The purities which could be obtained by the cadmium chloride method using a toxin and its corresponding toxoid have been compared, both before and after charcoal treatment. The results are given in Table IV. From this

TABLE IV.—*Showing the Purities Obtained by the Cadmium Chloride Precipitation of a Toxin and Its Corresponding Toxoid Before and After Charcoal Treatment.*

Toxin.

Eponit charcoal per cent.	Unpurified.			Amount of 5 per cent. $CdCl_2$ required per 100 c.c.	Purified product.		
	Lf.	T.N. mg./c.c.	Ratio* per cent.		Lf per mg. N.	Ratio* per cent.	Yield per cent.
None	38·5	2·14	37·5	15 c.c.	966	76	64
3	26·5	1·02	74	12 ,,	1520	97	62

Toxoid.

None	37·5	2·0	36	25 c.c.	563	53	67·5
5	23·5	0·632	92	12 ,,	1520	96	50†

* See Pope and Linggood (1939).
† Trace toxoid in supernatant, hence low yield.

table it will be seen that it is necessary to use more cadmium chloride to precipitate completely the crude toxoid than the toxin, and hence, the purity of the purified toxin is higher than that of the purified toxoid. It will be observed, also, that the "ratio" of the toxin is improved from 37 per cent. to 76 per cent. during the purification process, whereas that of the toxoid is only increased to 53 per cent. Thus it may be concluded that the formalization of proteins, proteoses, etc., present in the crude toxin has made the precipitation of the specific protein less selective in the case of the toxoid.

The purities obtained from the charcoal treated toxin and toxoid, by coincidence, are the same, owing to the fact that much more charcoal can be added to toxoid than to toxin without undue loss of Lf units, with the result that the purity, in terms of Lf units per mg. nitrogen, and also the "ratio" of the charcoal treated toxoid is higher than that of the toxin.

It is important to remember, therefore, when very high purities are required, to choose toxins and toxoids which yield, when treated with charcoal, products whose " ratio " is as high as possible. Particularly is this the case when purifying toxoid. With the latter, it should be possible to secure by charcoal treatment a " ratio " of 80 per cent. or higher, without undue loss of Lf units, if the growth of the organism on the broth is limited. If however growth has been excessive, i.e. heavy deposits have formed early on during the incubation period, it is probable that charcoal treatment will not result in a product whose " ratio " is high and subsequent purities will not be very good. The results given in Table II are not from selected toxins and toxoids and were purified irrespective of the growth of the organism, and in some cases growth was known to be excessive. It should be emphasized, also, that in all this work the brand of charcoal known as " Eponit " has been used. Recently other brands have been tried, many of which are not so good as " Eponit."

SUMMARY.

Eaton and Gronau's simplified method for purifying diphtheria toxin with cadmium chloride has been used, with some modifications, for the purification of both diphtheria toxin and toxoid made from tryptic digest broths.

A preliminary treatment of the toxin or toxoid with charcoal has resulted in higher purities up to 1500 Lf units per mg. nitrogen, i.e. 0·00067 mg. nitrogen per Lf unit.

Such a means of purification does not lengthen the flocculating time of the toxin or toxoid.

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CHANGES IN THE LUNG FOLLOWING INJECTIONS OF IODIZED OIL INTO THE TRACHEA.

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DURING a study of the post-operative complications of pulmonary lobectomy (Gowar, 1941) it became desirable to investigate the changes occurring in the lung after the introduction of iodized oil into the air-passages. Belsey (1937) had pointed out that massive collapse of the upper lobe of a lung after removal of the lower, appeared to occur more frequently in cases in which pre-operative bronchographic examination had been extensive than in those in which it had been limited. He suggested that the larger amount of iodized oil used might be the causative factor. He found that collapse was more common in those cases in which the interval between bronchography and lobectomy was less than eight weeks than in those with a longer interval. As a considerable amount of oil can usually be demonstrated in the lungs at the time of lobectomy, it was thought that its presence might in some way render the lobe more liable to collapse.

Earlier workers had reported changes in the lungs following the intrabronchial injection of iodized oil. Sato (1929) reported that the oil was absorbed by the bronchial epithelium, and found it in the cells of the bronchial cartilage. Kotatsu (1929) described proliferation of the bronchial epithelium with the formation of folds and adenomatous masses. Fried and Whitaker (1927) found desquamated epithelial cells and septal connective-tissue cells gathered together in certain areas to form tumour-like masses. Weinberg (1933) described massive atelectasis which, in some cases, was followed by pneumonitis, but it is probable that in his experiments a relatively large quantity of oil was injected and that a massive filling of alveoli resulted. In addition ether anaesthesia was used in his experiments. Sato, Kotatsu and Weinberg used lipiodol in their experiments, while Fried and Whitaker employed both lipiodol (Lafay) and iodopin (Merck).

It appeared from these reports that there might be a histological basis for the apparent greater frequency of collapse when lobectomy was performed shortly after bronchography, since the changes described might be expected to lead to a narrowing of the bronchial lumen.

EXPERIMENTAL TECHNIQUE.

Rabbits were selected for the work as they were easily obtained and kept in London, and their suitability for intravenous anaesthesia simplified the

technique. A solution of nembutal containing 1 grain per 1 ml. was injected slowly into the marginal vein of the ear in the proportion of 2/3 ml. per kilogram body weight. This produced anaesthesia sufficiently deep to permit the intra-oral manipulations, but the laryngeal reflex was retained and the animal usually recovered consciousness within two hours. Occasionally the anaesthetic resulted in respiratory failure, but as a rule the administration of artificial respiration led to a rapid re-establishment of normal rhythm, and fatalities were rare. The anaesthetized animal was secured on its back with the head extended, and under direct vision, using a head-lamp, a specially made tubular laryngoscope was passed down to the larynx. A No. 6 (French) rubber catheter was then passed between the cords during inspiration by means of an introducer. The catheter was inserted about one inch into the trachea and no attempt was made to reach its bifurcation, as it was thought that the size of the catheter relative to that of the bronchus might result in the oil being injected under pressure if it were passed into a main bronchus. The introducer and laryngoscope were removed and the animal turned on its right side. Two and a half or 3 ml. of neohydriol (May & Baker) were then injected into the trachea and distributed over the right lung by altering the posture of the animal. The catheter was now withdrawn and in the first group of animals a radiogram was taken to determine the distribution of oil. In the hope of confining the oil to the right lung the animal was laid on the right side until it recovered from the anaesthetic, care being taken to ensure that it had a clear airway. The passage of the catheter into the larynx and the subsequent injection of neohydriol usually caused a spasm of coughing, but this was of short duration and did not interfere with the operation. For a few days after the injection the animals were noticed to be off their food, but otherwise were well and usually had gained weight by the time they were killed.

EXPERIMENTS.

Three groups of rabbits received different treatment (see Table I).

Group 1.

Twenty-four rabbits were experimented upon during winter months (November, 1937, to February, 1938) and they were killed in pairs at intervals of 2, 4, 6, 8, 10, 12, 14, 18, 21, 28, 35 and 42 days after the injection of viscous neohydriol into the trachea. Two were killed by nembutal anaesthesia, one by a murderous attack from another rabbit and the remaining 21 by a blow on the neck. The trachea was not tied before the thorax was opened.

Group 2.

Subsequently, to clear up certain points which had arisen in connection with the first series, another 12 rabbits were injected in the summer months (July–September, 1938), 6 with viscous neohydriol and 6 with non-viscous neohydriol. They were killed, 11 by nembutal and 1 by a blow on the neck, at intervals similar to those in Group 1. In all the trachea was tied before the thorax was opened.

TABLE I.

Group 1 : Experiments in Winter Months.

Rabbit No.	Interval between injection and death (days).	Part of lung in which most neohydriol present.	Method of killing (B = blow on neck ; N = nembutal).	Trachea tied.
E. 7	2	Right lower lobe	B	No
E. 64	2	Right upper lobe	B	,,
E. 68	4	Right lung	B	,,
642	4	,, ,,	N	,,
E. 56	6	,, ,,	Died—trauma	,,
E. 86	6	,, ,,	N	,,
E. 57	8	,, ,,	B	,,
E. 70	8	Right upper lobe	B	,,
E. 61	10	,, ,, ,,	B	,,
E. 72	10	Right lung	B	,,
E. 3	12	Right upper lobe	B	,,
E. 54	12	Right lung	B	,,
E. 29	14	,, ,,	B	,,
E. 41	14	,, ,,	B	,,
E. 40	18	,, ,,	B	,,
E. 60	18	Right upper lobe	B	,,
E. 4	21	Not X-rayed	B	,,
E. 30	21	Right lung	B	,,
E. 20	28	Right lower lobe	B	,,
E. 27	28	Right lung	B	,,
E. 6	35	Not X-rayed	B	,,
E. 25	35	Right upper lobe	B	,,
E. 1	42	Right lung	B	,,
E. 11	42	Right lower lobe	B	,,
E. 53	3	Right upper and middle lobes	Died naturally	,,

Group 2 : Experiments in Summer Months.

836	2	Right upper lobe	B	Yes
835	4	,, ,, ,,	N	,,
814	6	Right lung	N	,,
784	8	,, ,,	N	,,*
813	10	,, ,,	N	,,
783	14	Right upper lobe	N	,,
782	18	Right lung	N	,,
781	21	Right upper lobe	N	,,
662	28	Right lower lobe	N	,,
644	35	,, ,, ,,	N	,,
661	42	Right middle lobe	N	,,
643	53	Right lower lobe	N	,,

* In this rabbit the tracheal ligature was inefficient and some air escaped from the lungs.

Group 3: Uninjected Control Rabbits.

	When killed.			
1	Summer months	—	N	Yes
2	Ditto	—	N	No
3	,,	—	B	Yes
4	,,	—	B	No
5	Winter months	—	N	Yes
6	Ditto	—	N	No

Group 3.

Six uninjected animals were used as controls. In the summer months 2 were killed by nembutal and 2 by a blow on the neck, and in one of each pair the trachea was tied before the opening of the thorax. In the winter months the remaining 2 were killed by nembutal, and in one the trachea was tied.

The lungs were removed immediately after death and fixed in 4 per cent. saline formaldehyde. Later, radiograms were taken to determine the amount and distribution of the oil remaining. The lungs were examined macroscopically and microscopically.

PATHOLOGICAL CHANGES IN THE LUNGS.

Atelectasis.

All the specimens from Group 1 showed variable degrees of atelectasis. The surfaces of the lungs were mottled, the partially or completely collapsed areas appearing brown, homogeneous and slightly sunken, while the remaining areas were, as a rule, pale yellowish-grey. A few white patches of emphysema were frequently present, especially in the anterior fringes. On section of the lungs complete atelectasis was rare in any part, since almost always air-bubbles could be squeezed with fluid from the cut surface, however collapsed the external appearance. Only in a few instances was the collapse of such degree that portions of lung tissue sank in water. Complete or incomplete atelectasis was frequently limited to a sub-pleural zone. The lower lobes were always more affected than the upper, and the left lower lobe was frequently more severely affected than the right, although the latter usually held the greater amount of neohydriol. The degree of collapse bore no relation to the interval which had elapsed since the injection of the oil. Microscopically partial collapse of alveoli was seen in all cases corresponding to the brown sunken areas seen by the naked eye. Complete collapse of alveoli was present to a much less degree.

In the 3 rabbits of the control Group 3 whose tracheas had been tied there was no atelectasis, while in the 3 whose tracheas had not been tied it was marked. In the injected animals of Group 2 the tracheas had been tied before the thorax was opened and atelectasis was absent from most, and only very slight in the remainder.

These results showed that the atelectasis was not due to the presence of neohydriol, but was probably due to escape of air from the lungs shortly before or after death. The distribution of the oil may have affected the distribution of atelectasis since the left lower lobe was frequently more affected than the right, which in almost all rabbits contained more oil. This could be explained by oil in the bronchial passages retarding or preventing escape of air.

Distribution of Neohydriol.

Radiograms taken immediately after the injection of neohydriol showed that as a rule the oil was most abundant in the right lung, particularly in the lower lobe (Fig. 1). Smaller amounts were distributed over the left lung. Further radiograms of the lungs taken after their removal from the thorax showed that a large amount of oil was still present (Fig. 2). In a number of rabbits, especially those killed shortly after injection, the post-mortem radiogram showed large globules of oil lying in the bronchi. It is well seen in Fig. 3, but it was unusual in an animal killed so long after injection. The appearance was not seen in any of the radiograms in Group 2, and it is suggested that opening the thorax without tying the trachea in animals of Group 1 allowed the lungs to collapse by escape of air, and at the same time the neohydriol flowed from the collapsing alveoli and smaller bronchioles into the larger air passages. As a rule most of the oil is eliminated from the lungs as a result of coughing, and the large amount remaining in the rabbit is probably due to the fact that this animal rarely coughs.

In the first group of animals the distribution of oil as seen in radiograms was confirmed by preparing frozen sections of the lungs. In Group 2 frozen sections were not made. The oil was present for the most part as large globules in alveoli, but some respiratory bronchioles and bronchioles were also filled. Only traces of it, as will be mentioned later, were intracellular. The oil stained brightly pink with nile blue sulphate and deeply with Scharlach R. In tissue fixed in osmic acid solutions the oil had a pale grey colour. When frozen sections of tissue fixed in solutions of osmic acid were treated with 95 per cent. alcohol, slight secondary or alcoholic reduction occurred so that the oil became a slightly darker grey. In tissue fixed in osmic acid the intracellular oil was only revealed by treatment with alcohol. The oil was entirely isotropic. It did not give a Schultz cholesterol reaction and was not present in Weigert-Pal preparations. These reactions differed from those given by neutral fat, such as occurs in adipose tissue. The latter was present in many of the sections of the lungs as fat emboli; it stained deeply with Scharlach R and pink with nile blue sulphate and did not give a Schultz cholesterol reaction. However it was readily blackened by osmic acid without subsequent alcohol treatment, and it was preserved and stained dark blue in Weigert-Pal preparations after about 2 months mordanting in Muller's fluid. In addition, anisotropic crystals were present in most of the globules of neutral fat. The fatty droplets present in the cells of the bronchial cartilages gave the reactions of neutral fat.

No evidence of absorption of neohydriol was seen other than its presence in a few cells as described later. The oil was visible to the naked eye in some

cases, the fluid expressed from the cut surfaces of the lungs showing oily globules. In one rabbit (E. 7) killed 2 days after the injection of oil the greater part of the right lower lobe was slightly enlarged, consolidated and pale yellowish-grey in colour. From the cut surface of this lobe a very greasy airless fluid exuded. This animal had received two anaesthetics, the first four days before it was killed, but an attempt to inject neohydriol then had failed owing to technical difficulties.

Formation of Epithelioid Cells.

In all the rabbits of Group 1, except 2 (E. 56 and E. 29) killed 6 and 14 days after the operation, groups of epithelioid cells were found scattered in the lungs. The groups were few and small in most rabbits killed less than 10 days after the operation, but in those with longer post-operative survival periods they were more numerous and larger, and often formed small nodules which reached up to 0·5 mm. in diameter (Fig. 4). In 5 of 10 rabbits killed 18 or more days after operation a few white sub-miliary nodules were visible under the pleura and they corresponded to nodules of epithelioid cells seen microscopically. The nodules were situated in alveolar walls or walls of respiratory bronchioles, and several contiguous alveolar walls were involved in the formation of one nodule. The cells encroached upon or obliterated the alveoli. Besides the cells in groups or nodules, single epithelioid cells were often present scattered in alveolar walls. The cells probably arose by proliferation and differentiation of fixed histiocytes of the interstitial tissue of the alveolar walls. The cells were round or polygonal and had an abundant rather basophil and slightly granular cytoplasm, which was stained purplish-blue with haematoxylin and eosin. Some cells contained vacuoles, and the majority contained finely particulate granules of black pigment. The latter resisted concentrated sulphuric acid and was probably soot. Frozen sections stained with Scharlach R showed that a minority of the epithelioid cells contained fat in the form of granules or small globules. It was probably neohydriol. One or more large extracellular globules of neohydriol were usually present in the nodules, and epithelioid cells lay around them. In some of the large nodules a few multinuclear giant cells were present and had cytoplasm and nuclei like those of the epithelioid cells. In four rabbits one or two such giant cells contained so-called radial inclusions (Fig. 5). These consisted of deeply eosinophil, refractive, crystalloid structures of fusiform shape and reached 10 by 2 μ in size. They were often slightly curved, and unlike crystals of most systems were isotropic. A large number were present in affected giant cells and they were arranged radially in a cart-wheel fashion or in a palisade. They were Gram-positive and frequently acid-fast. They were stained deeply by neutral red, Mallory's phosphotungstic acid haematoxylin and haematoxylin in the Loyez and Weigert-Pal myelin methods but not by Ehrlich's haematoxylin. They were not stained by Weigert's elastic stain, Scharlach R or nile blue sulphate and they did not reduce osmic acid or give reactions for free or masked iron. The structures were probably protein crystalloids. One nodule showed impregnation with material which was stained deeply by Ehrlich's haematoxylin and was present as granules, for the

most part fused together. It did not give Kossa's reaction. As it was not present in other sections further tests could not be applied. All the nodules of epithelioid cells were infiltrated with lymphocytes and eosinophil leucocytes, sometimes abundantly (Fig. 4). The nodules occurred in the various lobes roughly in proportion to the amounts of neohydriol shown therein in radiograms, and were not present in any lobe where the presence of oil had not been demonstrated.

In Group 2 nodules of epithelioid cells were present in the 6 rabbits injected with viscous neohydriol, but in only one of those injected with the non-viscous oil. The size and number of the nodules and the number of scattered epithelioid cells were less, sometimes markedly less, in the 6 animals of this group which had been injected with viscous oil than in the animals of Group 1 which had been injected with similar oil. These differences could not be explained.

In the control Group 3 no epithelioid cells were present. The formation of epithelioid cells was undoubtedly a reaction to the neohydriol, since such cells were not present in uninjected rabbits and their distribution corresponded with that of the oil. However, only a few of the cells contained oil. Unlike the viscous oil, the non-viscous form appeared to have little or no tendency to produce epithelioid cells. Viscous neohydriol is composed of iodized glycerine esters of the fatty acids of poppy-seed oil, while the non-viscous consists of iodized ethyl esters of the fatty acids.

Interstitial Pneumonia.

In all the rabbits some degree of interstitial pneumonia was present. In Group 1 it amounted to an infiltration with eosinophil leucocytes and lymphocytes of alveolar walls, walls of respiratory bronchioles, and the tissue around small arteries and rarely around bronchioles (Fig. 6). In 13 of the rabbits several small arteries or arterioles showed inflammation of the media or intima, or both, and in a few rabbits endophlebitis or phlebitis was also present. All the lobes of the lungs examined were about equally affected. In 2 rabbits killed 2 days after injection the pneumonia was more intense than in the

EXPLANATION OF PLATES.

FIG. 1.—Rabbit E. 72 (Group 1—killed 10 days after injection). Radiogram of chest immediately after injection showing neohydriol practically confined to the right lung.

FIG. 2.—Rabbit E. 72. Post-mortem radiogram of lungs. There is a large amount of neohydriol remaining in the right lung. Very little is present on the left side.

FIG. 3.—Rabbit E. 11. (Group 1—killed 42 days after injection.) Post-mortem radiogram of lungs; note the large amount of neohydriol still present, and the large globule of oil lying in one of the larger bronchi.

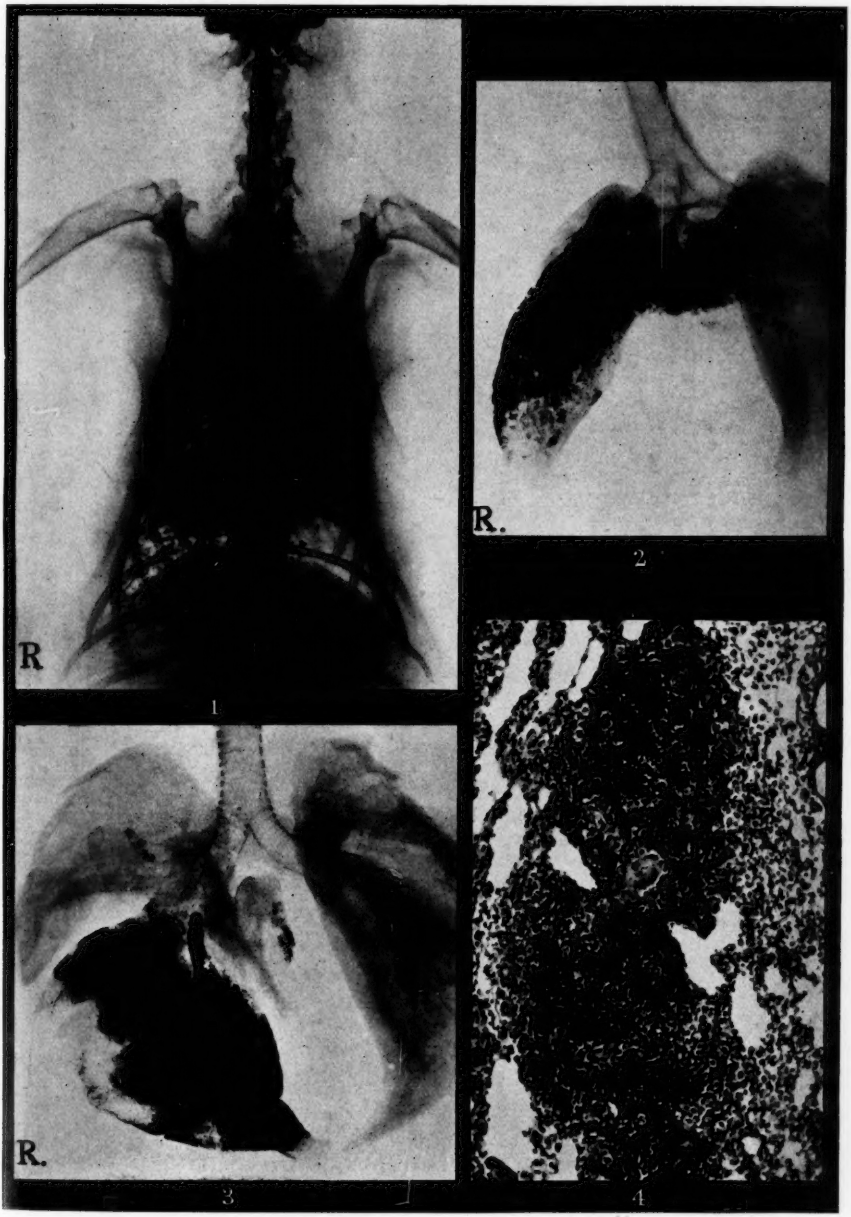
FIG. 4.—Rabbit E. 27. Lung: Nodule of epithelioid cells infiltrated with lymphocytes and eosinophil leucocytes. (H. and E. × 93.)

FIG. 5.—Rabbit E. 27. Lung: Radial inclusions in a giant cell in a nodule of epithelioid cells. (H. and E. × 480.)

FIG. 6.—Rabbit E. 7. Lung: Interstitial pneumonia around a small artery and extending into adjacent alveolar walls. (H. and E. × 135.)

FIG. 7.—Rabbit Control 4. Lung: Interstitial pneumonia around a small artery and extending into adjacent alveolar walls. Embolus of chiefly adipose marrow occluding the lumen of the artery. (H. and E. × 175.)

FIG. 8.—Rabbit E. 64. Lung: Large artery containing a mass of marrow, and A, a mass of cartilaginous matrix, and B, two small masses of hyaline cartilage. (H. and E. × 118.)



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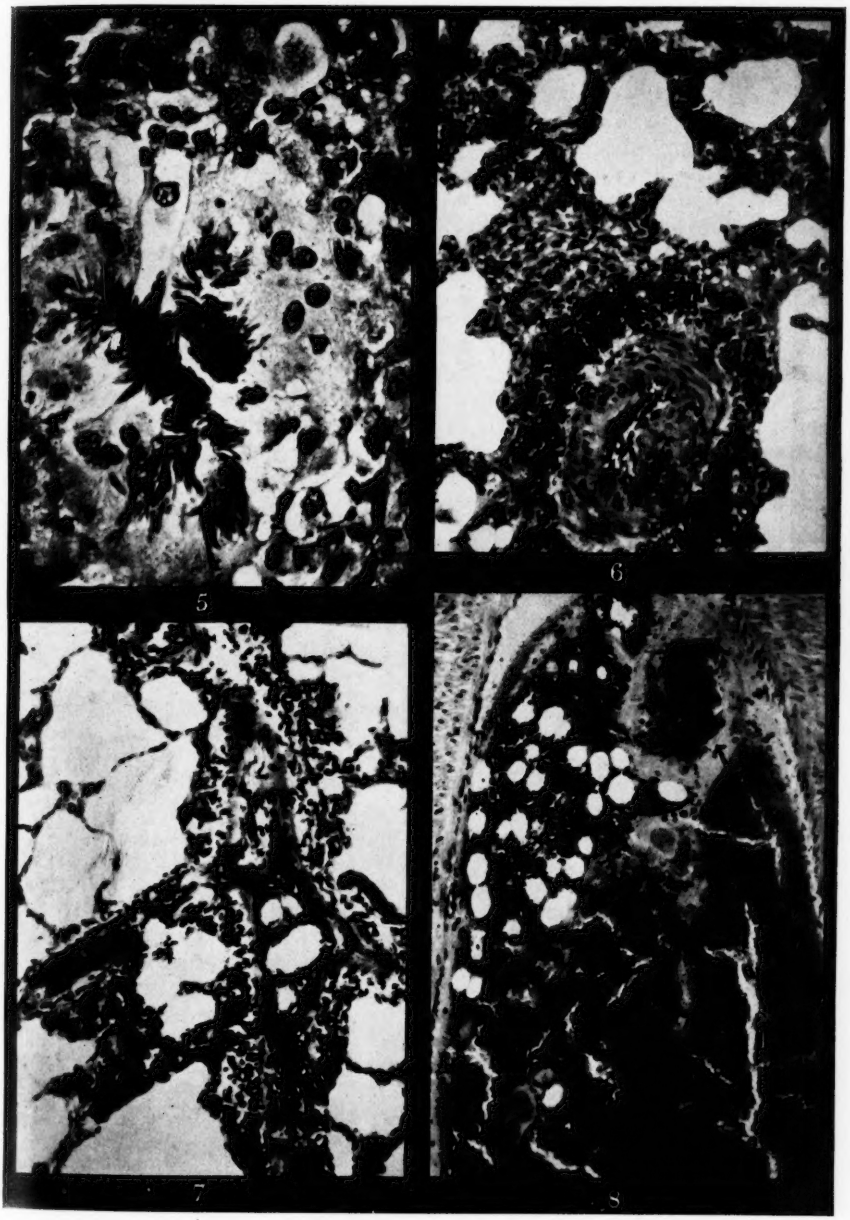

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remainder and the great majority of the inflammatory cells were eosinophil leucocytes. In those killed 4 days after the operation the pneumonia was slightly less extensive and intense and lymphocytes were relatively abundant. In most of the remaining cases the pneumonia was still less extensive.

In the rabbits of Group 2 which were killed during the summer months a mild similar pneumonia was present amounting to a very slight infiltration of the alveolar walls with eosinophil leucocytes and lymphocytes.

In 3 of the 4 rabbits of the control Group 3 which were killed during the summer months and in the 2 killed during the winter months a slight interstitial pneumonia was present similar to that in the rabbits of Group 2 (Fig. 7). In the remaining control rabbit it was more intense, and eosinophil leucocytes and lymphocytes infiltrated the connective tissue around many small arteries throughout the lung, and the alveolar walls in a narrow zone beneath the pleura, and in patches deeper in the lung tissue.

Because of the presence of interstitial pneumonia in the control animals and in lobes containing little or no neohydriol, it can be assumed that it was not directly due to the oil. In Group 1 but not in Group 2 it was more severe than in the controls. It is suggested that the pneumonia was due to the unsuitable environment of the animals during captivity, and its accentuation after the injection of neohydriol in Group 1 may have been due to an increased susceptibility to post-operative pneumonia during the winter months.

Embolism and Other Circulatory Changes.

In the 21 rabbits of Group 1 which had been killed by a blow upon the neck, fat embolism was present in the lungs. It could not be recognized in paraffin sections, but in frozen sections a variable number of arteries contained large globules of fat. It could most readily be identified by polarized light, since in almost all globules of fat solid crystals were present, whereas the neohydriol was entirely isotropic. In all but 6 of these rabbits marrow embolism was also present. The marrow was usually chiefly adipose and slightly haemopoietic, but in one it was entirely haemopoietic. It was usual to see one or a few small arteries occluded by marrow (Fig. 7), but in one or two rabbits marrow was also found free in large arteries (Fig. 8). In a few of these rabbits killed by a blow upon the neck, a few scattered megakaryocytes were seen filling capillaries in alveolar walls, or clumps of eosinophil leucocytes filled small arteries. These cells were probably embolic. In three of these rabbits embolism of cartilage occurred in the lungs. In one, small masses of hyaline cartilage and a mass of cartilaginous matrix lay free in the lumen of a large artery (Fig. 8). In the other two rabbits masses of fragmented, acellular matrix of cartilage plugged one or two small arteries.

In the two control rabbits which were killed by a blow upon the neck fat embolism occurred; in one megakaryocytes were present in alveolar capillaries, and in both there were clumps of eosinophil leucocytes in a few small arteries.

In none of the rabbits killed by nembutal was embolism present. It is therefore probable that the emboli were derived from the cervical vertebrae which were fractured as a result of the blow.

In all but 3 of the rabbits of Group 1 the lungs were congested. In 2 of

these, and in 2 others, haemorrhages were present. They were usually petechial, but in one animal larger haemorrhages were found. These changes were probably traumatic.

Oedema could not be appreciated macroscopically because the lungs were examined fixed. Microscopically, in many rabbits, scattered alveoli, respiratory bronchioles, bronchioles and bronchi contained a pale-eosinophil coagulum of oedema fluid. It was usually homogeneous in the alveoli, and indistinctly globular elsewhere. A few erythrocytes, lymphocytes or eosinophil leucocytes were occasionally present in the coagulum. This oedema was probably terminal and unrelated to the injection of oil.

In the anterior border of the lower lobe of the right lung of one rabbit (E. 3) of Group 1 killed 12 days after injection, the alveoli were filled with an oedema coagulum containing numerous fat phagocytes. In frozen sections the coagulum stained slightly with Scharlach R and contained free-cholesterol crystals, and the phagocytes contained cholesterol esters. The coagulum gave a slight diffuse Schultz reaction for cholesterol. The presence of the cholesterol was probably consequent upon chronic oedema, but it is doubtful whether or not this was due to the injection of neohydriol.

In each of 2 rabbits (E. 64 and E. 68) of Group 1 killed 2 and 4 days after the injection, a small artery in the lung was seen microscopically to be occluded by recent thrombus. The arteries were not inflamed. Fat and marrow embolism was present in each lung and the thrombosis may have been caused by stasis following embolism.

Other Findings.

In each of 2 rabbits (E. 6 and E. 20) a cyst was found loosely attached to the visceral pleura over the right lung. The larger measured 9 by 5 mm. At one end of each cyst there was a small white nodule about 1·5 mm. in diameter. The remainder of the wall was very thin, grey and translucent. In microscopic sections one was empty, the other contained a fluke in larval stage. Similar cysts were found in the peritoneal cavity in a number of rabbits.

In one rabbit (E. 11) there was a small nodule about 0·5 mm. in diameter in the lung. There was a central area of haematoxyphil structureless material and a peripheral zone of fibrous tissue infiltrated with lymphocytes. The haematoxyphil material gave a strong Kossa reaction and a very slight prussian-blue reaction. It was undoubtedly a healed inflammatory lesion, possibly tuberculous. In the same lung there was a small area, about 500 by 250 μ, of chronic interstitial pneumonia adjacent to a pulmonary artery. The alveolar walls were thickened, fibrotic and infiltrated with lymphocytes.

In the course of the experiments one animal (E. 53) of Group 1 died naturally 3 days after the injection of neohydriol. The lungs showed marked congestion and haemorrhage. The liver was large and yellow. There was severe fatty degeneration affecting all but the peripheral cells of the lobules. Many of the cells, chiefly those with fatty degeneration, showed hyaline necrosis. The portal systems were infiltrated with lymphocytes. The character of the fat was unusual; it stained deeply with Scharlach R, deep blue with nile blue

sulphate, and was readily blackened by osmic acid. In polarized light it was anisotropic, yielding fluid crystals on heating. The Schultz reaction for cholesterol was negative after 3, 7 and 14 days' treatment in iron alum, while a control human suprarenal gave a positive reaction in 7 days. The lipoid differed from neutral fat in being completely anistropic and in staining blue with nile blue sulphate. It differed from cholesterol esters in its reaction to nile blue sulphate and in its failure to give a Schultz reaction. We do not know of a similar lipoid in human pathology.

DISCUSSION.

After the introduction of neohydriol into the air-passages the only pathological change in the lung that could definitely be attributed to the oil was the presence of epithelioid cells which formed nodules. These nodules were much fewer after the injection of non-viscous neohydriol than after the injection of the viscous. Fried and Whitaker (1927) described how "desquamated (alveolar) cells and those which had migrated from the loose connective tissue of the alveolar septa gathered together in certain areas and formed tumour-like masses." These are apparently the nodules of epithelioid cells found by us.

We found very little evidence of phagocytosis of the oil. A little was present as granules or globules in a minority of epithelioid cells, but this was an inconspicuous change and foam cells containing oil were never seen. There was no evidence of removal of neohydriol from the lungs by macrophage migration. Fried and Whitaker (1927), Brown (1928), Kotatsu (1929) and Sato (1929), using various animals, described lipiodol phagocytosis as being marked. Fried and Whitaker (1927), Brown (1928), and Grill and Kling (1928) described the presence of lipiodol in the alveolar epithelial cells. Kotatsu (1929) and Weinberg (1933) stated that lipiodol was absorbed by the peribronchial lymphatics, and Brown believed that phagocytes transported the oil to the lymphatics and lymph glands. Kotatsu found fat globules in histiocytes in the hilar glands, spleen, liver and kidneys shortly after the injection of neohydriol into the bronchi. Sato (1929) described absorption of the oil by the bronchial epithelium and its presence in the cells of the bronchial cartilages. These observations are not in keeping with ours.

In all our rabbits some degree of chronic interstitial pneumonia was present and it was accentuated by the injection of oil in Group 1. The pneumonia was certainly not a reaction to the oil, and there was no evidence that the accentuation in the first group was dependent upon the presence of the oil. Brown (1928), Kotatsu (1929), Sato (1929) and Weinberg (1933) found that lipiodol produced an inflammatory reaction, but Fried and Whitaker (1927) and Grill and Kling (1928) could find none.

We found no evidence that the injected neohydriol produced areas of collapse, although Grill and Kling (1928), Kotatsu (1929) and Weinberg (1933) found such areas produced by oil obstructing the bronchi. Our experiments showed that atelectasis was prevented by ligature of the trachea before the thorax was opened, both in the injected and control rabbits. The distribution of the collapse that was always found in rabbits whose tracheas had not been

tied was probably influenced by that of the oil, since lobes containing most oil often showed less collapse than others containing little oil. Oil in the bronchi would tend to prevent the escape of air from the alveoli after opening the thorax.

We did not find any histological evidence to suggest that the presence of iodized oil would cause massive collapse of the remaining tissue of a lung after removal of one of its lobes. However it is possible that the state of collapse produced by opening the chest at operation might be maintained after the operation by oil obstructing the bronchi, or rendering the walls of the respiratory bronchioles and alveoli sticky and thus offering a resistance to re-expansion. Such operative collapse occurs only when the remaining upper lobe is free, and the presence of iodized oil in the lobe may therefore be one of the aetiological factors concerned in the greater incidence of massive collapse in such cases, as compared with those in which the upper lobe is adherent to the chest wall. As the quantity of oil present in the lobe diminishes with time, the presence of relatively larger amounts in cases with a short bronchography-lobectomy interval may explain why these cases are more liable to massive collapse than those with a longer interval. These points emphasize the desirability of operating with the upper lobe adherent, and of allowing sufficient time after bronchography for the oil to be coughed up and expelled from the air-passages, and the encouragement of such elimination by postural drainage.

We did not find any proliferation of the bronchial epithelium after a single injection of neohydriol, neither did Fried and Whitaker in their experiments. Kotatsu, however, after repeated injections of lipiodol found proliferation of the bronchial epithelium with the formation of papillary folds, producing appearances "resembling adenoma."

SUMMARY.

Rabbits received a single injection of neohydriol into the trachea and were killed at varying intervals afterwards.

Evidence has been given that when collapse of the lung was found it was due to another cause than the injection of oil.

The only definite result of the presence of the oil in the lungs was the formation of epithelioid cells in the alveolar walls, either scattered or forming nodules. Only a minority of these cells contained globules of oil. The distribution of the epithelioid cells corresponded with that of the oil. Multinuclear giant cells were present in some nodules and a few contained radial inclusions. The nodules were infiltrated with lymphocytes and eosinophil leucocytes. The non-viscous form of neohydriol produced many fewer epithelioid cells than did the viscous preparation. There was no histological evidence of absorption of the oil except by epithelioid cells. The oil could readily be differentiated from neutral fat, chiefly by its appearance in polarized light and by its reactions in preparations fixed in solutions of osmic acid.

In all rabbits, including uninjected controls, some degree of chronic interstitial pneumonia was present. It was probably due to their environment, but

it was accentuated by the operation of injecting the oil in the first group of animals.

In all rabbits killed by a blow upon the neck, but not in those killed by nembutal, emboli were present in the lungs. These were chiefly globules of neutral fat, but less frequently consisted of portions of marrow, small clumps of eosinophil leucocytes, single megakaryocytes or small masses of fragmented cartilaginous matrix or of hyaline cartilage. They were derived from the cervical vertebrae fractured by the blow.

It is suggested that any influence that the introduction of iodized oil into the air-passages may have on the occurrence of massive collapse of the lung is due to its mechanical action in predisposing to bronchial obstruction, rather than to histological changes resulting from its presence.

We wish to thank Mr. Tudor Edwards for his stimulating interest in this work. The first series of experiments was conducted in the Research Laboratories of the London Hospital, and the second series in the Bland-Sutton Institute of Pathology at the Middlesex Hospital, where we were indebted to Prof. J. McIntosh for providing the necessary facilities. Post-operative radiograms in Group 1 were taken in the Radiological Department of the London Hospital by courtesy of Dr. G. Vilvandré. For the radiograms of post-mortem specimens we are indebted to Mr. F. Watson, Senior Technical Assistant at the Buckston-Browne Research Farm, and for the photo-micrographs to Mr. John King of the London Hospital.

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THE WEIL-FELIX REACTION IN EXPERIMENTAL RAT-BITE FEVER.

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In this paper we report the unexpected finding that the sera of rabbits experimentally infected with rat-bite fever develop agglutinins for the OXK strain of *Proteus*. Moreover the incidence and the maximal titres attained are much higher than in experimental tsutsugamushi disease (syn. Japanese river fever). The OX19 strains of *Proteus* are not agglutinated. This OXK type of serological response has hitherto been regarded as pathognomonic of the tsutsugamushi disease or of infections by *Proteus OXK* bacilli.

During an investigation of several strains of tsutsugamushi in laboratory animals throughout the past nine years certain data obtained as a result of accidental contamination by the *Spirillum minus* had suggested this finding, but only recently have we been able to study strains of this spirillum that could be regarded as free from suspicion of concomitant infection by a typhus-like disease ; and we have delayed publication accordingly.

In order to establish the authenticity and purity of our recently isolated strains of rat-bite fever, we report their investigation in detail that would not otherwise be justified ; although certain of our findings, notably concerning the immobilization test, are new.

THE WEIL-FELIX REACTION.

The introduction of this reaction in 1916 as an important aid in the diagnosis of typhus fever by Weil and Felix, and its amplification by Fletcher and Lesslar (1926) that revealed the world-wide distribution of the typhus-like fevers, are fully described in the literature of typhus fever. Felix and Rhodes (1931) investigated the antigenic relationship between the classical X19 strains and the XK strain of Fletcher and Lesslar ; and concluded that the XK strain is a variant derived from an X19 culture, that it represents another type of *Proteus X*, and proposed the symbols HXK and OXK for its H and O variants respectively.

" Main " and " Group " Agglutinins.

The XK type agglutinins present in sera from man and certain laboratory animals convalescent from tsutsugamushi have been classified by Felix (1933) as " main " agglutinins, agglutination of other *Proteus* strains, viz. X2 and X19, being absent or insignificant. In the exanthematic fever of Sao Paulo

XK type agglutinins occur in man and rabbit in titres much lower than do the X19 type ; and Felix concludes that they are " group " agglutinins, due to " group " antigens present in the virus. In the three varieties of typhus fever in South Africa XK type agglutinins appear in the serum of man and rabbit in titres which, though usually lower than those of the X2 and X19 agglutinins that also occur, do occasionally exceed them ; Felix (1935) and Pijper and Crocker (1938) conclude that the agglutinins of these three types are " group " agglutinins, and that the " main " antigen in the virus is yet to be discovered.

THE AGGLUTINATION OF "OXK" STRAINS OF PROTEUS IN EXPERIMENTAL RAT-BITE FEVER.

Lewthwaite and Savoor (1936*a*, 1936*b*) isolated two strains of a typhus-like fever from rats trapped in endemic areas. Both strains were maintained with ease in guinea-pigs, and gave the usual criteria of experimental infection. Later, a concomitant infection of the guinea-pigs with the causal organism of rat-bite fever, the *Spirillum minus*, was found. A similar experience has been reported by others (Chuiton and Moreau, 1933 ; Suzuki, 1934).

The Weil-Felix reactions in rabbits experimentally infected with these two strains were as follows : Of the first strain, 17 of 22 rabbits gave positive reactions, agglutination being only of the OXK type ; the titres in 4 exceeded 1/500, one attaining 1/1700. Of the second strain, 34 of 56 gave positive reactions, agglutination being only of the OXK type in the majority, but of the OX19 type in a considerable minority ; OXK titres in 8 exceeded 1/500, one attaining 1/1925. The proportion of positive reactions in the second strain approximated to that usually found by these authors in the investigation of experimental tsutsugamushi in the rabbit (50–60 per cent.). That for the first strain was much higher, a result the more striking since maintenance of this strain in the rabbit proved to be impossible ; for, of 13 rabbits inoculated by the intra-ocular route, only 2 developed the ocular reaction, and that feebly, that is typical of tsutsugamushi virus, and it could not be reproduced on passage ; yet 8 gave a positive Weil-Felix reaction of the OXK type, 2 to very high titres. The titres given by both rat strains were approximately twice as high as those given in rabbits by strains derived from human cases of tsutsugamushi. The *Spirillum minus* itself caused great enfeeblement in all rabbits, and death in most. In a dual infection by rat-bite fever and typhus-like fever the enfeeblement should be still greater ; and, in consequence, one might reasonably expect the serological response, if due to the latter virus alone, to be correspondingly feebler than in an uncomplicated infection with this virus. But paradoxically, higher titres in both strains and an increased incidence in one strain were obtained. It seemed possible that this enhanced serological response might somehow be attributed to the *Spirillum minus per se*.

As attempts to separate the two viruses failed, study of each was not possible ; and further investigation of the problem was deferred until strains of rat-bite fever uncomplicated by any suspicion of a typhus-like infection could be studied.

A PROBABLE STRAIN OF *SPIRILLUM MINUS* RECOVERED FROM GUINEA-PIGS INOCULATED BY A SUSPENSION OF TICKS.

During 1936, in an attempt to send strains of the Malayan typhus fevers to America for comparison with the many typhus strains there available, we fed batches of the American wood-tick, *Dermacentor andersoni*, on guinea-pigs experimentally infected with tsutsugamushi or urban typhus. These ticks, which have not been found in Malaya, were sent to us from America for this purpose. From ticks retained by us we could obtain no conclusive evidence of transmission of these viruses by them. The rest of the ticks were sent to our collaborator in the project, Dr. R. R. Parker, Director of the Rocky Mountain Spotted Fever Laboratory, Montana, U.S.A.; and from one batch of these he obtained in guinea-pigs a typhus-like infection, the nature of which was obscure. For comparative study Dr. Parker attempted to send this strain to us; and seven batches of normal ticks were fed by him on guinea-pigs infected with the strain, and despatched to Malaya. We re-fed each batch of ticks on normal guinea-pigs, ground them up, and injected the suspensions severally into seven pairs of normal guinea-pigs. No evidence of a typhus-like infection could be obtained. But one guinea-pig thus inoculated developed an infection that was characterized by fever, Strauss' reaction, enlargement of the spleen, and the presence in the blood and tunica exudate of a spirillum indistinguishable from the *Spirillum minus*—a syndrome typical of experimental rat-bite fever. This "Montana" strain we maintained serially in the guinea-pig without difficulty. No peritoneal exudate was found, nor were Rickettsia demonstrable in smears of the exudate on the tunica vaginalis or spleen. Cultures of heart-blood were made on ordinary laboratory media; no *Proteus* or other organism was found. Infected blood was inoculated intraocularly into 9 rabbits; only one showed an ocular reaction, but the signs were not typical of experimental tsutsugamushi, and could not be reproduced on passage, nor did the animal acquire an immunity to tsutsugamushi. Successive Weil-Felix tests were made on 7 rabbits; high agglutination of the OXK strain of *Proteus* developed in 6, but none for the OX19 strain. Maximum titres were respectively 1/1700 on the 28th day after inoculation; 1/1000 on the 22nd day; 1/800 on the 24th day; 1/440 on the 28th day; 1/340 on the 28th day; and 1/110 on the 15th day, on which day this rabbit died. These titres are much higher than those usual in passage rabbits of strains of tsutsugamushi derived from human cases (Fig. 2).

Thus, apart from serology, the experimental disease produced by this "Montana" strain lacked the pathognomonic criteria of experimental tsutsugamushi. Our earlier suspicion was strengthened that an infection of rabbits by the *Spirillum minus* can cause the development of agglutinins for the OXK strain of *Proteus*.

Whether this strain had been transmitted by the ticks or had originated in the guinea-pig into which they had been injected cannot be known. In our experience of some thousands of guinea-pig passages we have never known a contaminating rat-bite fever infection to originate in a guinea-pig. The spirillum may have been present in the ticks; although Dr. Parker did not find it in ticks of the same lots retained by him for experiment.

In view of the possible though very remote connection of this strain with that tsutsugamushi strain on infected guinea-pigs of which we had fed the original batch of ticks, suspicion that the ticks might have acquired a typhus-like infection could not be rigidly excluded. We therefore awaited the opportunity to study uncomplicated human cases of rat-bite fever. Three such, named respectively the "Dicum," "Ipoh" and "Loke" strains, have now been studied.

HUMAN CASES OF RAT-BITE FEVER.

The "Dicum" Strain.

A Tamil, admitted to hospital in Kuala Lumpur under the care of Dr. Dicum, had been bitten on the forearm by a rat eight days previously.

Fever ensued six days after the bite was inflicted. At the site of the bite was an oval ulcer, about $\frac{1}{2}$ in. by $\frac{3}{8}$ in. in size; the edges were raised, oedematous, and of irregular outline; the base was covered by granulation tissue, there being no scab. The epitrochlear gland of the arm was enlarged, but no other gland. The spleen was not palpable; heart and lungs were normal; there was no rash. On the day after admission the temperature fell to normal by crisis, so that the first bout of fever had been of three days' duration. On the third day after admission, serum from the edge of the ulcer failed to reveal the suspected *Spirillum minus*; and guinea-pigs inoculated with blood at the same time failed to show any reaction. On the following day fever recurred, ushered in by a rigor and accompanied by headache, and a rash appeared papular in character and fading on pressure, over the angle of the mouth and jaw of one side; examination of blood-smears for *Spirillum minus* proved negative, but two guinea-pigs were inoculated intra-peritoneally with blood, and from these a strain of rat-bite fever was established. The second bout of fever lasted for five days; it was terminated by injections of N.A.B., after which the ulcer healed rapidly; and the patient, after nine days' uneventful convalescence, was discharged from hospital.

The experimental disease.

The results of the Weil-Felix reaction and the immobilization test in the various laboratory animals are detailed in a later section.

Guinea-pigs.—The "Dicum" strain has been maintained serially for 16 months by the intra-peritoneal inoculation of 94 guinea-pigs with infected brain, spleen, blood, or washings of the testis. The features of the infection have not differed materially from those described by previous workers (Robertson, 1924; Theiler, 1926; McDermott, 1928).

That certain features of the experimental disease may differ appreciably with different strains of the *Spirillum minus* has been noted by others; for brevity we omit detailed comparison of our strain with other recorded strains.

A febrile reaction occurred in 79 per cent., after an incubation period that averaged 10 days, with limits of 6–18 days; its main feature was its irregularity, fever of varying duration alternating with remissions. The infection was fatal within 3 months in 95 per cent. Of 55 guinea-pigs not used for passage, 40 survived for one month, 12 for two months, and 3 for three months.

Of 44 males, 26 developed a swelling of the scrotum 4–5 days after onset of fever, though wider limits were occasionally noted. The scrotal reaction differed from that of experimental urban typhus in that an exudate in the tunica sac was absent or scanty, and adhesion of the testis to the scrotum the rule. Ano genital inflammation was not seen, although it was common in the spirillum infections that contaminated the rat strains referred to earlier in this paper. Prolonged infection brought progressive emaciation, alopecia, conjunctivitis and keratitis with mucoid discharge, and the formation of small crusted ulcers near the nostrils and eyelids. Post-mortem examination revealed that the spleen first became enlarged but later smaller; ascites was absent; the axillary and groin glands were hyperaemic and enlarged.

The *Spirillum minus* was first found in blood smears between the 8th–23rd days; thereafter it was constantly present, in numbers that, though fluctuating from day to day, increased as the infection progressed to the fourth week, after which they diminished. When in maximum number, 3 or 4 were found in each field; on one occasion 15 per field were present.

The morphology of the spirillum agreed with the published descriptions. One or more flagella were present at both ends; the maximum number seen at one end was three. The spirillum stained readily by Leishman's or Giemsa's method; to demonstrate flagella required prior fixation by the vapour of osmic acid or formalin.

White rats.—Six rats were inoculated intra-peritoneally with virulent material; all became infected. Spirilla were found in the blood from the 17th day onwards, but in much smaller number than in the guinea-pig. The rats showed no clinical sign of infection during six months' observation; but persistence of infection was proved by its transmission to guinea-pigs by an inoculum of splenic tissue.

White mice.—Twenty-eight mice have been used. Spirilla appear in the blood as early as the 5th day after inoculation, in approximately the same concentration as in guinea-pig blood. The mice are otherwise healthy, and procreate normally; the young do not show spirilla in their blood. The concentration of spirilla, greater than that in the blood, reported by Das Gupta (1938) to occur in the peritoneal exudate, was not found in our mice; aspiration of the peritoneal cavity secured no more than 0·05 c.c. of fluid, in which spirilla were sparse. During six months' observation infected mice have remained healthy, though still harbouring spirilla. Since infection of the mouse is so easily effected, maintained and detected, we used this animal throughout our investigation as an indicator of the successful infection of other species of laboratory animal.

Rabbits.—Twenty-five rabbits were inoculated with material from infected guinea-pigs, 2 intra-peritoneally with a suspension of brain and spleen, 22 intra-ocularly with blood, and 1 intra-ocularly with aqueous humour from an atypically reacting rabbit. The intraocular route was preferred, since all infections of rabbits with the virus of tsutsugamushi are made by this route; the ocular and serological response due to each virus would thus be more strictly comparable. The only visible sign of infection was progressive emaciation; the iridocyclitis that is typical of experimental tsutsugamushi infection was not seen; one atypical ocular reaction occurred, but could not

be reproduced on passage. The infection is better tolerated by the rabbit than by the guinea-pig ; of 16 that have died, 4 died during the 1st month, 1 during the 2nd, 3 during the 3rd, and 8 during the 4th–6th months ; 1 has survived for 7 months.

Spirilla can be detected in the rabbit from the 10th day onward by the inoculation of blood into mice. To demonstrate their long persistence, the blood of 2 rabbits that died 10 and 17 weeks respectively after infection was inoculated on the day of death into two mice, numbered correspondingly 10 and 17 ; spirilla were found in mouse 10, though not in mouse 17. It may happen that the organisms cannot be detected in the peripheral blood of the rabbit, as indicated by abortive search of the blood of an " indicator " mouse, yet can be demonstrated to have been present in the rabbit tissues. Thus a rabbit the blood of which had been found repeatedly to be negative for spirilla by mouse inoculation was killed, and two mice inoculated, one with blood and one with a suspension of brain and spleen ; successive examinations of blood from the former mouse were negative, but were positive in the latter from the 9th day onwards.

Monkeys.—Four macacus monkeys were inoculated intradermally and subcutaneously with blood plasma from infected guinea-pigs ; dark-ground examination showed the inoculum to abound in spirilla. After 9–12 days fever occurred, but was fitful ; in one it lasted for one day only ; in another, for three days ; in the other 2 an initial rise for one day was followed some days later by another brief rise. A moderate degree of leucocytosis occurred in all, in contrast to the leucopenia that is given by monkeys infected experimentally with tsutsugamushi. None of the monkeys showed a dermal lesion at the site of inoculation. That these 4 monkeys were infected was shown by inoculations of their blood into control guinea-pigs, and by the positive results of the immobilization test.

Man.—An attempt was made to study the serological response in three human volunteers. The 1st and 2nd were suffering from tabes dorsalis, the 3rd had partially recovered from a hemiplegia ; the Wassermann and Kahn reactions were weakly positive in the 1st, and strongly positive in the others. Inoculations were made by both intradermal and subcutaneous routes. Febrile reaction was fitful in one patient and absent in the others. A rash appeared in one only, on the 19th day ; its elements were macular, irregular in outline, faded on pressure, and situated on the abdomen, chest, back and forearm. At the sites of intradermal inoculation an erythema developed, that in two of the patients progressed to the formation of a blister, with subsequent ulceration in one. At the sites of subcutaneous inoculation an extensive erythematous induration developed in all three, but no ulceration. Proof of the successful infection of the 2nd and 3rd patients was given by the immobilization test.

The "Ipoh" and "Loke" Strains.

The patient from whom the " Ipoh " strain was derived was bitten by a rat. The wound healed in 4 days ; 11 days later it broke down and fever developed. On the 3rd day of fever his blood was inoculated into mice.

Blood-films from these were repeatedly examined for the *Spirillum minus*, were negative on the 5th–8th day after inoculation, but positive on the 12th day.

The second patient (" Loke ") was bitten on the hand by a rat that she was trying to catch. The wound healed, but later broke down, with resultant ulceration, cellulitis and lymphangitis, and accompanying fever. Mice inoculated with serum from the edge of the ulcer failed to show spirilla ; but in mice inoculated with blood drawn during the febrile reaction, spirilla were found 10 days later.

The two strains were maintained in mouse and guinea-pig without difficulty. Clinical signs of infection in these animals and in the rabbit were identical with those noted for the " Dicum " strain.

TWO STRAINS ISOLATED FROM WILD RATS.

By the courtesy of Dr. J. Coutts Milne we were enabled to isolate two additional strains of rat-bite fever from rats trapped in the town of Kuala Lumpur. Their isolation was effected as follows :

From each of 42 rats heart-blood was drawn, defibrinated, centrifuged at low speed, and the plasma examined under the dark field. Spirilla were detected in 2 ; whereupon white mice and guinea-pigs were inoculated with blood and splenic tissue from one, with blood only from the other.

These two strains, respectively named the " Milne " strain and the " Coutts" strain, were maintained serially in mouse and guinea-pig. The clinical signs in these animals and in the rabbit, and the morphology and pathogenicity of the causal spirilla, were identical with those noted above for the human " Dicum " strain.

THE SEROLOGY OF EXPERIMENTAL RAT-BITE FEVER.

We investigated the Weil-Felix reaction and the immobilization test in our laboratory strains, the former in five strains, the latter in three strains.

The Weil-Felix Reaction in Rat-Bite Fever.

The methods and criteria used were those described by Lewthwaite and Savoor (1936*b*). In particular, an OXK titre below 1/50 was regarded as negative ; a titre between 1/50 and 1/75 as inconclusive unless the form of the curve of successive titres showed the waxing and waning features characteristic of active infection, and a titre greater than 1/75 as positive. With few exceptions, samples of blood were drawn from animal and man before inoculation, and subsequently at intervals of approximately 5 days from the 10th–12th day onwards. Both living and alcohol-treated suspensions of *Proteus X* strains were used.

The "Dicum" strain.

Rabbits.—Of 25 rabbits tested, the sera of 24 gave positive agglutination with the OXK strain of *Proteus* ; none gave any agglutination with the OX19 strain. In 2 the maximum titres were 1/96 and 1/110 respectively ; in 3 they

lay between 1/200–1/300 ; in 5 between 1/300–1/400 ; in 2 between 1/400–1/500 ; in 3 between 1/500–1/600 ; in 3 between 1/800–1/900 ; in 2, 1 and 3 rabbits they were 1/1000, 1/1700 and 1/3400 respectively.

Maximum titres were attained on the following days : in 3 rabbits on or before the 15th day after inoculation ; in 7 between the 15th–20th day ; in 6 between the 20th–25th day ; in 5 between the 25th–30th day ; in 2 and 1 on the 40th and 50th days respectively. Indicating the rapid rise of agglutinins in the serum, positive titres were attained not later than the 15th day in 16 rabbits, the 20th day in 22.

In 8 rabbits a spontaneous secondary rise of agglutinins occurred ; it was especially marked in one (Fig. 1).

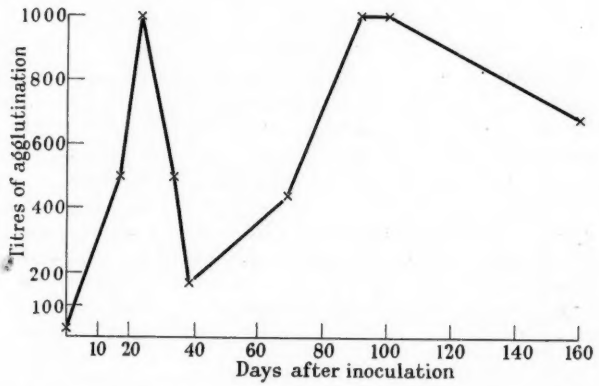

FIG. 1.—Spontaneous secondary rise of OXK agglutinins in a rabbit.

This curve (see also Fig. 3) resembles that given by Topley (1933) to illustrate the fluctuation of agglutinins for *B. typhosum* observed in relapsing cases of typhoid fever. These secondary rises we consider to be due to the long persistence of the spirilla in the infected rabbit.

White rats.—The results of the Weil-Felix reaction in the only 3 infected white rats tested were as follows :

	Titre of OXK-type agglutinins.			
	(0.)	(17.)	(50.)	(69.)
Rat 1	0	0	85	110
Rat 2	0	0	85	50
Rat 3	0	19	85	50

(Figure in brackets = day after inoculation.)

No agglutinins developed for the OX19 strain of *Proteus*. These results were entirely consistent with, though less striking than, the corresponding results in rabbits.

Monkeys.—In sharp contrast to the findings in rabbits, the Weil-Felix

reactions of the four macacus monkeys used were far from conclusive ; in 2 the serum showed no rise of agglutinins for the OXK strain of *Proteus* ; in 2, a slight rise. Agglutination of the OX19 strain was entirely absent in 3 ; in the fourth some agglutination for this strain did develop, but not to a conclusive titre.

Further investigation of the Weil-Felix reaction in this animal is in progress.

Man.—Three volunteers were tested. The reaction was negative in two throughout 39 and 68 days' observation respectively ; in the third there was a rise in agglutinins for the OXK strain of *Proteus* to a titre of 1/110, which in man cannot be regarded as conclusively positive.

These results in monkey and man need to be extended before conclusions can be drawn ; they are given here only by reason of the sharp contrast they afford, inadequate though they be, with the corresponding serological results of our rabbit experiments.

The "Ipoh" Strain.

The sera of 4 rabbits were tested, with similar results, maximum titres for OXK being 1/250, 1/1000, 1/96 and 1/250 on the 17th, 23rd, 22nd and 25th days respectively.

The "Loke" strain.

The sera of 3 rabbits were tested, again with similar results, maximum titres for OXK being 1/220, 1/170 and 1/125 on the 23rd, 21st and 32nd days respectively.

The "Milne" strain.

The sera of 3 rabbits were tested. All gave early positive agglutination of the OXK strain, maximum titres being 1/500, 1/500 and 1/125 on the 20th, 30th and 15th days after inoculation respectively.

The "Coutts" strain.

The sera of 3 rabbits were tested, with similar results, maximum titres for OXK being 1/3000, 1/96 and 1/300 on the 25th, 25th and 15th days respectively.

To sum up the Weil-Felix reaction in rabbits infected with these 5 strains, 3 of human origin and 2 of rat origin, the sera from 36 of 37 rabbits tested agglutinated the OXK strain of *Proteus*, the titres showing the waxing and waning features that indicate an active infection. Agglutination of the OX19 strain was entirely absent. The above proportion of positive reactions is much higher than that obtained by us in rabbits experimentally infected with tsutsugamushi strains (55 per cent.).

OXK Type Agglutination in the Sera of Rabbits Variously Infected.

We have compared our agglutination results in rabbits infected by the "Dicum" strain with those obtained in rabbits infected by (*a*) the "Montana" strain, reported earlier as being probably a strain of rat-bite fever ; (*b*) two

stock strains of tsutsugamushi ; (*c*) rabbits infected with cultures of *Proteus OXK*. In each strain successive titres plotted for each animal were reduced to a composite curve (Fig. 2).

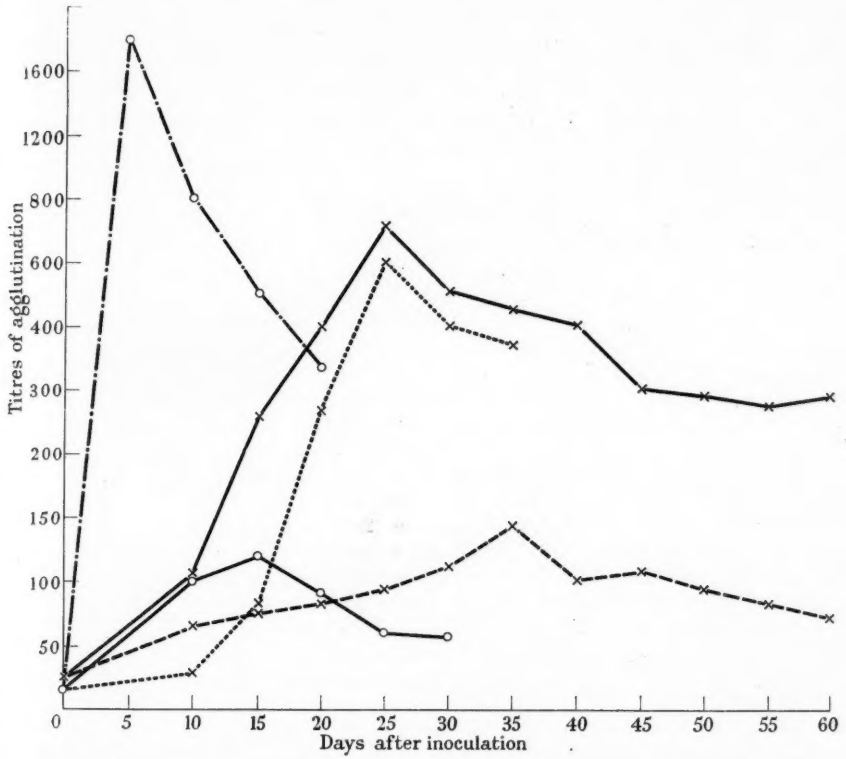

FIG. 2.—Shows composite graphs of the Weil-Felix reactions of rabbits respectively infected as indicated ; and illustrates the differing height and time-relations of the titres obtained.

– · – ○ – ·	Proteus OXK bacilli	7 rabbits.
———X———X	Dicum strain of rat-bite fever	19 ,,
· · · · X · · · · · X	Montana strain of rat-bite fever	6 ,,
– – – – X – – – – – X	Raub strain of tsutsugamushi	13 ,,
———○———○	Seerangayee strain of tsutsugamushi	6 ,,

Salient features in this graph are :

(a) "*Montana*" *strain.*—The curve is almost coincident with that of the Weil-Felix reactions from rabbits of the "Dicum" strain. The maximum titre of 1/600 was reached on the 25th day after inoculation.

(b) *Tsutsugamushi strains.*—The "Raub" curve differs from that of the rat-bite fever strains in two particulars, viz. the rise is more gradual and the maximum titre much lower (1/160 on the 35th day as compared with 1/725

on the 25th day). The "Seerangayee" curve shows an early rise of titre, but to a lower maximum (1/125 on the 15th day).

(c) *Proteus OXK*.—The rabbits were inoculated by the intra-ocular route with 0·1 c.c. of a 6-hour culture of *Proteus OXK* on an agar slope that had been suspended in 20 c.c. of saline. The samples of sera tested included some drawn on the 5th day after inoculation. This curve differs from those of (*a*) and (*b*) strikingly, in that there is an immediate and steep rise which reaches its maximum (1/2000) on the 5th day to decline at once to 1/800 on the 10th day, 1/500 on the 15th day, and 1/330 on the 20th day, so that the curve has the outline of an inverted letter V.

The Immobilization Test.

Based on the presence of "spirillicidal" or "immobilizing" bodies in the serum of infected animals and man, this test was first described by Ido, Ito, Wani and Okuda (1917), and is accepted as a reliable criterion of infection with the *Spirillum minus*. Our technique, which follows in essentials that of the above workers, is as follows:

Blood from an infected guinea-pig is defibrinated, and spun at 500 r.p.m. for ten minutes; cells, platelets and fibrin are thus eliminated, and the plasma found to contain the spirilla in concentrations that may attain 30–50 per field. To a drop of this suspension of spirilla is added a drop of the test serum in various dilutions. One drop of the mixture is examined, immediately and after 15 minutes, under the dark-field, using a 1/7th in. oil-immersion lens.

The test, made at room temperature (28° C.), is considered positive if motility has ceased by the end of an arbitrary time-limit of 15 minutes. Insomuch as we obtained positive reactions in dilutions as high as 1/400, our modified technique appears to have increased the sensitivity of the test as compared with the procedure used by Ido and other workers.

The following sera of varied origin were used as controls: 22 human sera from healthy adults, 22 human sera from adults with positive Wassermann and Kahn reactions, 12 from normal guinea-pigs, 16 from normal rabbits, 12 from normal white rats, and 2 proprietary antileptospirosis sera. All gave negative immobilization tests, motility persisting unimpaired for at least two hours. In contrast, when undiluted strongly positive serum of rat-bite fever strains was used, loss of motility was very rapid, in some cases almost instantaneous. Swift direct progression of the spirilla gave place to hesitant backward and forward movements, and these in turn to a rapid spinning *in situ*: this ended, the spirilla faded from vision, apparently in a state of granular disintegration. Clumping does not occur. Inactivation at 56° C. in no way diminishes the spirillicidal action of the serum.

Dicum strain."—Rabbits, monkeys, human volunteers, white rats and guinea-pigs were infected and tested.

From 5 of the 25 rabbits used, serum was drawn at 5-day intervals for 60 days, and the titres of immobilizing bodies plotted (Fig. 3). Sera from the other 20 rabbits were tested less often; all were positive, maximum titres being in many as high as 1/400.

That the serum of 4 monkeys gave negative or inconclusive Weil-Felix

reactions has been reported above; the same sera, however, gave positive immobilization tests with the infecting spirillum, 3 in a dilution of 1/100, 1 in a dilution of 1/400.

Results similar to those from the monkeys were obtained with sera from the 3 human volunteers, which also had given negative or inconclusive Weil-Felix reactions; on the 32nd day after infection the sera from 2 gave positive immobilization tests in a dilution of 1/100, while the serum from the third was positive on the 28th day in a dilution of 1/2 but negative in 1/10.

Sera drawn from 3 white rats two months after infection were all positive, 2 in dilutions of 1/100.

Sera from 5 guinea-pigs were tested, at varying periods after infection, against spirilla from guinea-pigs and mice infected with human or rat strains. All gave a negative immobilization test, a finding contrary to that of McDermott (1928).

"Milne" and "Coutts" strains.—Sera from 3 infected rabbits of each strain were tested. All gave positive results.

The Immobilization test with sera containing OXK agglutinins.

In view of the positive agglutination of the OXK strain of *Proteus* that is common to tsutsugamushi and to experimental rat-bite fever in rabbits, special interest attached to the results of these tests.

Expt. 1.—Immobilization tests were made on sera drawn during convalescence from 8 human cases of tsutsugamushi; each had given positive OXK-type agglutination to titres that exceeded 1/2000. The immobilization test proved to be completely negative in 6 of the sera; in 2 it was positive in a dilution of 1 : 2 and 1 : 4 respectively, but negative in both in a dilution of 1 : 8.

Expt. 2.—Sera were drawn and tested from rabbits convalescent 4–5 weeks after infection with laboratory strains of tsutsugamushi (4) and Sumatran mite-fever (2) (these diseases we consider to be identical—Lewthwaite and Savoor, 1940); the OXK-type agglutination had been respectively 1/100 in two, 1/220 in two, 1/250 in one, and 1/1000 in one. The immobilization test was negative in all.

Expt. 3.—Sera were tested from 5 human cases convalescent from the urban endemic typhus of Malaya, a disease which according to serological classification is X19 in type; all had agglutinated the OX19 type of *Proteus* to high titres. The immobilization test was negative in all.

Expt. 4.—Four normal rabbits were immunized against an OXK strain of *Proteus*; from these, samples of sera that agglutinated this strain to titres that exceeded 1/5000 were tested; the immobilization test was negative in all.

Expt. 5.—Two normal rabbits were immunized against an HXK strain of *Proteus*; samples of sera that agglutinated the strain to titres that exceeded 1/5000 were tested; the immobilization test was negative in all.

Three strains of Spirillum minus *compared by the immobilization test.*

Sera drawn from rabbits of the "Dicum," "Milne" and "Coutts" strains at a point when the immobilization titres were known to be high were tested

against the homologous and heterologous strains of *Spirillum minus*. The titres are tabulated below :

Rabbit serum.	Source of strain.	Spirilla from guinea-pig.		
		Dicum.	Coutts.	Milne.
Dicum	Man	1/300	1/350	1/300
Coutts	Rat	1/200	1/200	1/250
Milne	Rat	1/250	1/250	1/200

These figures indicate that, taking the immobility test as a criterion, the strains isolated from man and rat are antigenically the same. They suggest that the " Dicum " strain is of rat origin ; a finding that supports an earlier experiment designed to elucidate this point, in which 8 of 38 sera from town rats, when tested against the spirillum of the " Dicum " strain, were found to be spirillicidal.

COMPARISON OF THE DEVELOPMENT OF OXK AGGLUTININS AND OF IMMOBILIZING BODIES IN EXPERIMENTAL RAT-BITE FEVER IN THE RABBIT.

Five rabbits were infected with the " Dicum " strain ; samples of serum were drawn on the day of inoculation, and later at 5-day intervals from the

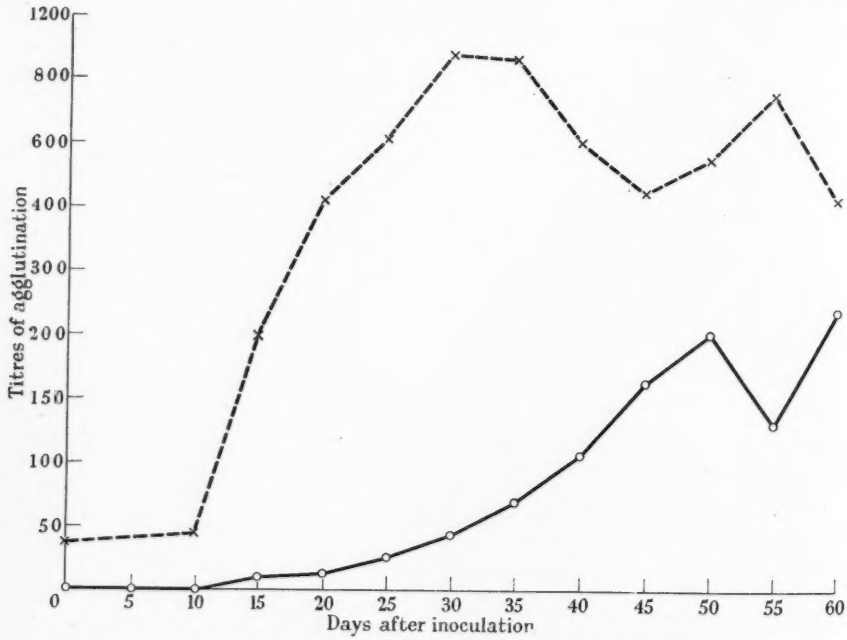

FIG. 3.—Composite graph of Weil-Felix and immobilization titres from 5 rabbits, comparing development of these two types of antibody.

----X---- Titres of OXK agglutinins.
——⊙—— Titres of immobilizing bodies.

5th day to the 60th day. Composite curves of the OXK agglutinins and immobilizing bodies respectively in each sample of serum were plotted (Fig. 3).

Salient features are :

(1) The rise of OXK agglutinins begins much earlier and progresses much more rapidly than does the rise of immobilizing bodies.

(2) In both a partial decline in titre is succeeded by a secondary rise. The secondary rise of immobilizing bodies lags behind that of the OXK agglutinins ; this time-lag is approximately equal to the primary lag of the initial rise of titres, and to the time-lag separating the attainment of maximum titres in each curve. The decline and secondary rise of titres in the curves are, then, approximately parallel. It would appear from these features that, the immobilizing spirillicidal bodies being admittedly " true " antibodies, the parallelism of the two curves indicates that the antigen responsible for the development of the OXK agglutinins is an integral part of the antigenic mosaic. It may well be that the recurring time-lag at various points in the development of the two types of antibody finds its explanation in a differing disposition in depth of the two antigenic components.

ABSORPTION TESTS.

Two sera from convalescent rabbits of the " Dicum " strain were absorbed by *Proteus OXK* bacilli, as follows : To 1 c.c. of a suspension of the bacilli was added 1 c.c. of a 1 in 5 dilution of the serum. Absorption proceeded for four hours at 37° C. and then for 18 hours at 4° C. The results are given below.

	OXK titres.				Spirillicidal titres.	
Serum.	Before absorption.		After absorption.		Before absorption.	After absorption.
	alc.	liv.	alc.	liv.		
1	1/800	1/1000	0	1/125	1/20 = +	1/20 = +
					1/50 = 0	1/50 = 0
2	1/250	1/880	0	1/220	1/20 = +	1/20 = +
					1/50 = 0	1/50 = 0

alc. = alcoholized cultures ; liv. = living cultures.
+ = immobility test positive ; 0 = immobility test negative.

All the OXK agglutinins for the alcoholized cultures, and the majority of those for the living cultures, were absorbed. In contrast, the titres of the immobilizing antibodies in both sera remained unchanged. The converse absorption tests were found to be impracticable, since a sufficiently concentrated suspension of spirilla could not be obtained ; attempts at culture of the spirilla on various media, including the chorio-allantoic membrane of the chick embryo, proved unsuccessful.

THE PURITY OF THE RAT-BITE FEVER STRAINS.

To uphold our view that the OXK type agglutination of *Proteus* given by rabbits infected with rat-bite fever is due entirely to infection with the *Spirillum minus*, it is essential to exclude any possibility of there having been a concomitant infection with strains of tsutsugamushi or with *Proteus OXK* organisms.

Absence of Tsutsugamushi Infection.

This could be excluded on clinical evidence and by cross-protection tests.

Clinical evidence.

Earlier in this paper (p. 275) we have recorded the isolation from wild rats of two strains of a typhus-like fever that subsequently showed a contaminating infection with the *Spirillum minus*. The signs of infection caused by both typhus-like fever and contaminant were readily recognizable.

Because of that experience we have since sought diligently for any evidence of a concomitant tsutsugamushi infection in four of our strains of *Spirillum minus*. None has shown any of the signs of experimental tsutsugamushi. Thus in the guinea-pig ascites and Rickettsia were absent. In the rabbit the typical ocular reaction and Rickettsia were never found. White mice infected with these strains did not die, in contrast to the corresponding findings in tsutsugamushi in which infected mice do not survive beyond 10–14 days. In 4 monkeys inoculated intradermally with the " Dicum " strain no dermal lesion occurred, whereas in 20 monkeys and gibbons similarly inoculated with strains of tsutsugamushi some degree of dermal lesion was invariable ; leucocytosis was the rule, in contrast to the leucopenia of tsutsugamushi infections.

By cross-protection tests.

These comprised :

(1) Cross-infection of rabbits with one or other of three strains of rat-bite fever and two stock strains of tsutsugamushi, the criteria of infection, where applicable, being the recurrence of an ocular reaction and the restimulation of OXK type agglutinins.

(2) Cross-infection tests in the guinea-pig.

Expt. 1.—Eleven rabbits that had reacted with high titre OXK agglutination to infection with the rat-bite fever strain were reinoculated by the intraocular route after the titres had fallen to normal limits, 2, as controls, receiving the homologous strain, 9 receiving the stock strains of tsutsugamushi. The homologous strain failed to produce re-stimulation of agglutinins. The tsutsugamushi strain evoked in all 9 rabbits the full evolution of the ocular reaction that is typical of experimental infection with that strain ; in 4 of these 9 successive Weil-Felix reactions were done, but none showed re-stimulation of agglutinins, though only one survived re-inoculation long enough to allow the 60 days of observation necessary before the Weil-Felix may be regarded as negative in infections with tsutsugamushi strains.

Expt. 2.—In a converse experiment, one rabbit, that had reacted typically with ocular reaction and high OXK agglutination to an infection with a tsutsugamushi strain, was re-inoculated with a rat-bite fever strain ; OXK agglutinins were re-stimulated to a high titre.

Expt. 3.—Six guinea-pigs immune to the " Seerangayee " strain of tsutsugamushi were re-inoculated with a rat-bite fever strain ; all reacted fully.

Expt. 4.—In order to attempt the converse experiments in guinea-pigs, prior treatment with arsenic was essential because of the persistence of the spirilla. Three guinea-pigs showing the extreme signs of prolonged infection

were treated with mapharside, three doses intraperitoneally at 4-day intervals. Progressive improvement of condition, with regrowth of the hair and clearing of the eyes, was soon evident. Spirilla, hitherto found at will, disappeared from the blood within 24 hours of treatment. When fully convalescent four months later they were re-inoculated with approximately 10 minimal infective doses of tsutsugamushi virus. All three reacted fully, dying of the infection.

Thus by both clinical and serological criteria cross-protection was absent.

Absence of Proteus *OXK Organisms.*

At first, simple routine blood-cultures were made from 9 guinea-pigs infected with one or other of the three rat-bite fever strains, "Dicum," "Coutts" and "Milne," 1 c.c. of blood being inoculated into 10 c.c. of broth. All remained sterile. Later we used the more exacting method recommended by Felix (1933) for the isolation of *Proteus X* strains from patients suffering from classical epidemic typhus, the relevant excerpt from which is as follows:

"Blood is collected into a number of sterile tubes, not less than 10 c.c. per tube, and kept at 37° C. for two to three hours, until complete coagulation takes place. As soon as possible the separated serum is removed from the coagulum by simply pouring it off. This procedure is repeated once or twice, until the coagulum is left almost free from serum. Some of the tubes are then incubated aerobically at 37° C. without adding any nutrient medium whatever; the other tubes are left in the dark at room temperature. To protect the tubes from drying they are covered with rubber caps or kept in an incubator containing a wet atmosphere. At intervals of one or two days several large loopfuls, taken from the inner part of the coagulum, are inoculated into serum broth or blood-broth and also on the surface of serum-agar and blood-agar. The blood and serum for these media should be from normal guinea-pigs or rabbits. If the subcultures taken from the coagulum in the course of ten to fourteen days still remain sterile, some serum-broth or plain broth is added to the coagulum and incubation and subculturing are continued for several weeks. While all the broth tubes are incubated for several weeks the agar plates are only kept for twenty-four hours at 37° C. and remain subsequently at room temperature for ten to fourteen days. Successful cultivation from the blood can only be expected during the late period of incubation and in the course of the very first days of fever."

This technique was adhered to rigidly. Cultures were made from the heart-blood of guinea-pigs and rabbits, and from the spleen of guinea-pigs. As media, serum-broth and blood-agar were used.

Guinea-pigs.

Two pairs of guinea-pigs were infected with the "Dicum" strain of rat-bite fever. On the day on which the *Spirillum minus* was first found in their blood, i.e. on that day on which such blood has proved consistently to be infective to the rabbit with resultant development of OXK agglutinins to high titres, 5 c.c. of heart-blood were drawn from both animals of each pair, and mixed and cultured as above. From one pair, blood was again drawn six days later and similarly cultured. All the large number of cultures and subcultures proved to be sterile.

One guinea-pig of the "Coutts" strain was killed 10 days after spirilla had first been found in the blood. Ten c.c. of blood were cultured by the above technique; all cultures were sterile.

Spleens from three infected guinea-pigs of the "Dicum," "Milne" and

" Coutts " strains were put into broth. At intervals of 2 days three cultures taken from the cut surface of each spleen were made on blood-agar, as recommended by Felix ; observation for 10–14 days showed them to be sterile.

Rabbits.

Two pairs of rabbits were inoculated with blood (sterile on culture) from an infected guinea-pig of the " Dicum " strain. All in due course developed OXK agglutinins. Five c.c. of blood were drawn from each rabbit on the 4th and 12th day after inoculation from one pair, on the 8th day from the other, in order to embrace the late incubation period and early period of infectivity as judged by the rise of OXK type agglutinins to positive titres. The blood of each pair was pooled and cultured according to Felix's technique ; all cultures were sterile.

The Reaction of the Normal Guinea-pig to Inoculation with Proteus OXK *Bacilli.*

To determine this, we inoculated 8 guinea-pigs intraperitoneally with 0·1 c.c. of a thin suspension of the bacilli. In 2 there was no febrile reaction ; in 6 a moderately high fever was present next day, and was continuous, though declining gradually, for three days ; thereafter the temperature remained normal. In the absence of an incubation period it is presumed that this febrile reaction, early and brief, was due to toxic absorption. In support of this was the finding that blood cultures made on the 10th, 15th, 20th and 25th days from 4 of the 8 guinea-pigs proved to be sterile. It would appear, therefore, that guinea-pigs do not suffer from an infection due to *Proteus OXK* bacilli. That *Proteus OX19* bacilli do not produce disease in guinea-pig and rabbit is well known.

Throughout this investigation the blood which caused the serological reaction in rabbits has been drawn at random from infected guinea-pigs during a period varying from the 10th to the 53rd day after inoculation. If the presence of a contaminating infection with *Proteus OXK* be postulated to account for the high Weil-Felix reactions given by the rabbit sera, then it should have been possible to isolate *Proteus OXK* bacilli from the blood of infected guinea-pigs during that period, i.e. there should have been found a prolonged bacteriaemia. But, as noted earlier, all attempts to effect this failed ; which accords with our experience that even the inoculation of *Proteus OXK* bacilli is not followed by invasion of the blood stream, at least from the 10th day onwards.

SUMMARY AND CONCLUSIONS.

The suspicion that strains of rat-bite fever in experimentally infected rabbits give a Weil-Felix of the OXK type arose from our study of two murine strains of typhus-like fevers found to be contaminated with the *Spirillum minus*, and of another strain thought to be a typhus strain but which proved to be infected only by this same spirillum. Later, study of 3 human and 2 murine strains of this disease from which all suspicion of a contaminating

typhus-like infection of *Proteus* infection could be excluded gave confirmatory evidence, rabbit sera consistently yielding high titres of OXK type.

Three of these strains were studied intensively for evidence of contamination with *Proteus* bacilli; none was found. Moreover the inoculation of normal guinea-pigs with *Proteus OXK* bacilli was not followed by an infection or by a bacteriaemia. It is evident therefore that the presence of this bacillus in the infected guinea-pig inocula that provoked the high OXK agglutinins in the rabbit could be excluded.

None of the five strains showed any evidence of infection with tsutsugamushi virus.

The high proportion of reacting rabbits (37 out of 38) tested from all five strains is in itself sufficient to indicate that the OXK type agglutination obtained is due to the *Spirillum minus per se*.

Three men suffering from tabes dorsalis, and four macacus monkeys, were infected experimentally with the *Spirillum minus*. Proof of successful infection was given by the positive immobilization test; yet the Weil-Felix reaction was entirely negative in two of the men and two of the monkeys, and the slight rise in titre that occurred in the others could not be regarded as positive by our standards. This series in man and monkey is, however, far too small to warrant conclusions being drawn.

Study of Fig. 2 indicates that the average Weil-Felix titres given by sera from groups of rabbits infected respectively with *Proteus OXK* bacilli, rat-bite fever and tsutsugamushi attain widely differing maxima. Whereas titres from the first-named group are very high, those from tsutsugamushi are low; those from the rat-bite fever groups occupy an intermediate position.

We consider that the "OXK" antigen no less than the "spirillicidal" antigen is an integral part of the antigenic mosaic, and that these two components may well have a different disposition in depth; since composite curves plotting the development of the antibodies produced by these two antigens show a close parallelism in initial rise, decline and secondary rise.

High titre sera from rabbits immunized against cultures of *Proteus OXK* and *HXK* give consistently negative immobilization tests. Moreover, when the OXK type agglutinins in sera from rabbits of a rat-bite fever strain were absorbed by *Proteus OXK* organisms the titre of immobilizing bodies remains unchanged. Since the immobilizing bodies are the spirillicidal bodies and therefore the "main" or "specific" antibodies, the OXK agglutinins of rat-bite fever sera must be considered to be "minor" or "group" antibodies.

Castaneda and Zia (1933) investigated the Weil-Felix reaction given by convalescent sera from cases of classical and Mexican typhus; as a result of absorption tests with *Proteus OX19* organisms and the causal Rickettsia they concluded that the OX19 agglutinins were minor agglutinins. It is probable that the OXK agglutinins in convalescent tsutsugamushi sera will similarly be found to be minor agglutinins, contrary to the views of those who consider the viruses of the typhus group to be the parasitic stage of the X strains of *Proteus*.

The demonstration of a positive Weil-Felix reaction of OXK type in the sera of rabbits infected with rat-bite fever indicates that an OXK component is present in an organism, the *Spirillum minus*, that is entirely unconnected

with Rickettsia in general and with *R. tsutsugamushi* in particular. It may be that further investigation will show that the nature of this common antigenic factor is a polysaccharide, as has been shown to exist by Castaneda (1935) in the case of the *Proteus OX19* bacillus and the Rickettsia of murine typhus.

It is of interest to recall the findings of Felix (1933) in experiments with London rats, in which he states that " O agglutinins of relatively high titres for one or the other X type were often found in the serum of normal tame rats "; and that " the agglutination reactions obtained with *Proteus X* strains and the serum of wild or tame rats ought to be interpreted with utmost caution." According to a personal communication from Dr. Felix, agglutination titres of 1/500 for the strains OX19 and OXK have been observed in normal tame rats of London stock. It is justifiable to infer from our findings that the OXK type of agglutination obtained by him may have been due to an infection of the rats by the *Spirillum minus*. For whereas no case of typhus of the OXK type in man or rat has yet been reported in London, many cases of rat-bite fever with isolation of the causal *Spirillum minus* have been recorded.

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